THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

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MSS. for the successive numbers of Vol. 27 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICE.

KARL SPIRO.

(1867–1932.)

KARL SPIRO was born in Berlin on the 24th of June 1867. He studied chemistry first in Berlin, and subsequently in Greifswald and Würzburg, obtaining the Degree of Ph.D. He followed up his chemical training by studying medicine, at Breslau, Berlin, Strassburg and Leipzig, obtaining his M.D.

After a short period in an industrial capacity, he went to Strassburg in 1892 as assistant to Schmiedeberg, but soon became assistant to Hofmeister in the Physiologisch-chemisches Institut, with whom he worked for nearly 25 years.

In 1919, when the French took over Alsace, he was forced to vacate his post in Strassburg, and as no suitable position was at that time available in Germany, he migrated to Switzerland, where he obtained the post of Pharmacologist to the chemical firm, Sandoz, in Basle. In 1921, he was appointed Professor of Physiological Chemistry at Basle University, on the death of Prof. Bunge, and held the chair at the Physiologisch-chemische Anstalt until the time of his death in March 1932.

To give a detailed account of Spiro's work would be no easy matter. In the first place, his activities as a research worker covered an unusually wide field, and secondly, a considerable proportion of his work is published under the names of his assistants and students. The latter fact reflects the modesty which was characteristic of the man—as one of his assistants, writing in the *Kolloid Zeitschrift* has put it—"Brachte man aber am Schlusse der Arbeit den ihm gebührenden Dank an, so wurde er sicher in der Korrektur durchgestrichen!"

In general, his activities were devoted to the study of the chemical and physical properties of protoplasm: *e.g.* the part played by buffers in intracellular reactions, osmotic pressure effects, and subsequently the colloid systems of the cell. Shortly before his death, he was, in conjunction with others, compiling a book entitled "Die Kolloidik in der Medizin," which unfortunately he has not lived to complete. He also did a considerable amount of work on protoplasmic toxins and the effect of various organic compounds on the cell. Being a student of Emil Fischer, his biological work was built on a very sound knowledge of organic chemistry, in fact his early training in pure chemistry is evidenced throughout his work on physiological problems.

The problems of *intra-vitam* staining also received his attention and in later years he worked on the physiology of pain.

As a pharmacologist, his activities led to the production of "Pyramidon," "Panthesin-Balsam" and numerous other medicaments.

An idea of his very widely varied activities can be gained from the fact that a reference list of his published work in the *Kolloid Zeitschrift* [1932, **59**, 257] covers 5 pages of close type.

He was joint editor of *Ergebnisse der Physiologie* and also of *Jahresbericht über die gesamte Physiologie*, and was on the editorial staff of the *Biochemische Zeitschrift*. He was a member of the Biochemical Society (1927) but never

published anything in the *Journal*, his work appearing entirely in the German and Swiss journals.

Prof. Spiro was a man of very attractive personality and quite outstanding character, which much endeared him to all who had the good fortune to work under him, either as students or assistants. The philosophical teachings of Goethe were of great interest to him, and a source of considerable inspiration.

His death was realised as a great loss to research workers both in Germany and Switzerland, and a sad personal loss to all who had the good fortune to know him by personal contact.

He died suddenly from heart-failure on March 21st, 1932, leaving a widow and two sons.

H. T. F.

I. THE CHEMISTRY OF THE PRODUCTS OF *COCOS NUCIFERA*. II.

By JUAN PEDIGE CHARLES CHANDRASENA.

From the Chemistry Department, Ceylon University College.

(*Received September 9th, 1932.*)

As a preliminary to the investigation, which is now in progress, of the cellulase and lipase present in the haustorium of *Cocos nucifera* it was thought that an examination of the properties of the "milk" of the nut, in which the haustorium develops during germination, would indicate the optimum conditions, particularly p_H, under which the enzymes act. It was also thought that it would be of interest to examine at the same time the variations, if any, in the properties of the liquid as the nut matures, especially since it not only persists but increases during all the stages of growth of the nut.

Bunch No.	Sp. gr.	p_H	Total solids %	Ash % of total solids
11	1·014	5·1	4·01	18·99
10	1·014	4·9	4·29	14·76
9	1·016	4·8	4·66	12·95
8	1·021	5·0	5·66	12·09
7	1·025	5·2	7·31	9·94
6	1·021	5·3	6·17	11·12
5	1·018	5·4	5·16	11·76
4	1·018	5·1	5·38	11·58
3	1·014	5·1	4·65	13·01
2	1·019	5·0	4·72	14·05
1	1·015	4·8	4·83	11·77

During germination.

No. of weeks	Sp. gr.	p_H	Total solids %	Ash	Weight of haustorium g.
0	1·015	4·8	4·83	11·77	0
10	1·015	5·6	4·42	12·83	1
11	1·014	5·6	4·27	13·13	9
12	1·014	5·6	4·61	12·60	16
13	1·013	5·6	4·16	12·38	1
14	1·014	5·6	4·62	11·84	21
15	1·008	5·5	2·90	14·30	68
16	1·015	5·3	3·92	11·37	78
17	1·020	5·9	5·02	11·74	3

Nuts from the youngest to the oldest bunches were obtained from each of six palms from various parts of the Island. Those of the oldest bunches were put away to germinate and the "milk" examined at intervals.

The results, one typical series of which is given on p. 3, show the following properties.

(1) The temperature of the liquid immediately after picking the nut is about 1° below air temperature although the nut is exposed to direct sunlight.

(2) The specific gravity varies in the different sets but increases in each with age.

(3) p_H varies between 4·6 and 5·6 but the value for each set is nearly constant, not changing with age, in spite of the decrease of mineral matter (shown by ash %).

(4) Even while germination proceeds p_H remains constant and is nearly the same as the optimum for *Ricinus* lipase observed by Willstätter and Waldschmidt-Leitz.

II. INVESTIGATIONS INTO VITAMIN A-FREE BASAL DIETS. I.

By ALFRED LOUIS BACHARACH.

From the Glaxo Research Laboratory, Osnaburgh Street, London, N.W. 1.

(*Received November 18th, 1932.*)

Two of the main difficulties associated with the use of diets for work on vitamin A have been the occasional failure of experimental animals to reach a "plateau" of inhibited growth—to "run out" of vitamin A, in the phrase of certain investigators—and rather more frequent failure of the animals, after they have ceased to grow, to respond to even relatively large doses of the vitamin with anything approaching normal growth performance. It is generally held that the first difficulty is due to high vitamin reserves, the result of a too liberal pre-experimental (including pre-natal) diet and that the second is due to some inadequacy in the basal diet, since, if this is deficient in other constituents besides vitamin A, the mere provision of the latter will not bring about continued normal growth.

Most of the published work that bears on these problems has appeared incidentally in papers dealing with the detection or estimation of vitamin A, and there have been relatively few investigations into the experimental technique as such. Notable exceptions, however, are the papers by Coward and her colleagues [1929, 1, 2], who have studied amongst other things especially the effect of different sources of protein (caseinogen) on the ability of the animals to resume growth when vitamin A is restored to their diet. Mention must also be made of the very interesting critical study recently published by de Lourero [1931], who has examined and discussed all the chief constituents generally present in vitamin A-free diets. A recent communication by Culhane [1932] was concerned with the regularity of the incidence of xerophthalmia in relation to the source of protein in the basal diet, but the details of her work have not yet been published. Moore [1932] has also made passing reference to similar problems.

These problems are of considerable importance, quite apart from their theoretical interest, since a multiple deficiency in the basal diet might quite conceivably cause an over-assessment of the amount of vitamin A in a substance that was being compared with a simple solution of carotene in vegetable oil. Further, the question of securing uniform and rapid response to a diet deficient only in vitamin A is of as immediate concern to all workers in the field as is that of securing quick and normal response to curative treatment.

Although our interest in this problem was partly stimulated by Coward's findings, we have for years been attempting to approximate more closely to a theoretically perfect basal diet without complete success, and we are presenting here in a condensed form such of our results as seem either to throw light on the unsolved problems or to call for some explanation that we ourselves have been unable to find.

Various preliminary experiments, undertaken as time and the necessary stock of animals were available, indicated an inadequacy of commercial yeast extract (marmite) as a source of the vitamin B complex, since better growth was obtained either by increasing the amount of the extract or by supplementing the diet with wheat germ; in this we have confirmed the findings of several other workers. The preliminary experiments also suggested that the weight of the animals when they begin to receive the basal diet is a less important factor in determining their running-out age or weight than is their age. In this we find ourselves in agreement with Nelson [1928], who has advocated transferring the nursing mother and sucklings to the basal diet some 16 to 18 days after the birth of the litter. Further, our experiments indicated that, if this is done, the replacement in the stock diet of a New Zealand dried milk, with its relatively high vitamin A content, by an English winter-dried milk has little, if any, effect on the running-out time. These observations tally well with some work recently published by Dann [1932]. Again, the time of survival of animals on a vitamin A-free diet, their initial growth rate and the time of reaching a growth plateau do not appear to be significantly influenced by the presence or absence of fat in that diet.

The animals employed in this work were exclusively of the albino variety of the Norwegian rat (*Mus norvegicus*); all were descended by the closest in-breeding from three bucks and six does obtained in the winter of 1923 from the Wistar Institute of Anatomy and Biology, Philadelphia, U.S.A. All animals were removed by at most one generation from animals that were themselves the product of continuous brother-sister mating of descendants from the original nine animals. We have never followed the practice advocated by some workers of reducing the numbers of all litters after birth to 6 or 7. The mothers are allowed to bring up as many of their young as they are able; this has once been as high as 17, and the average size of litters at weaning has been 6·7.

In all the work discussed in this paper we have, unless otherwise stated, compared only isogenic animals (animals of the same sex and from the same litter).

For the last three years we have fed our general breeding stock (those, that is, whose F_1 litters alone have been used in these investigations) on a diet constituted as follows:

Whole-meal flour	70 parts
Full-cream New Zealand dried milk...	30 ,,
Commercial yeast extract (marmite)	5 ,,
Water	45 ,,

Lettuce (about 2 g.) or other green stuff is fed daily, liver or lean meat (about 2 g.) twice weekly, and fish (a few g.) once a week.

This diet, which is a modification of Sherman's stock diet B [Sherman and Burtis, 1928], was introduced to replace a diet of mixed kitchen scraps, on which our whole stock had previously been fed, since this was not always available in sufficient amount and must in any event have varied considerably in its content of fat-soluble vitamins; the scrap diet, supplemented with various sources of vitamins and minerals, is now used only for a selected number of special pairs bred as a "genealogical" stock to produce the general breeders; its replacement does not appear in any way either to have reduced the numbers of young animals born or to have affected their health.

In spite, however, of the uniform diet and the intensely inbred nature of the animals, there is still a large, unexplained, and uncontrollable variation in

the response of individual animals to basal diets. Many animals receiving a diet on which some animals cease growing at about 120 g. weight (males) in 8 to 10 weeks will continue to grow for months with no evident signs of any kind of hypovitaminosis (see negative control animal, Fig. 1). Others cease growing at a relatively high weight, 180 g. or more; their growth response to any supplement is then limited by the normal growth rate for that weight. Some animals appear to stop growing for as long as 14 days and then spontaneously continue to grow again for 4 or more weeks, behaving exactly as if a vitamin supplement had been administered, though actually no known change whatever has been made in their diet. The literature abounds in graphs showing animals that have been

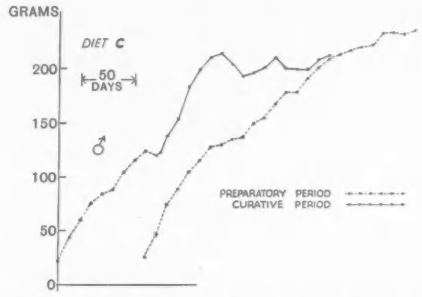

Fig. 1. Anomalous growth curves of animals on vitamin A-free diet; no cessation of growth in negative control animal and secondary growth plateau in animal receiving curative treatment.

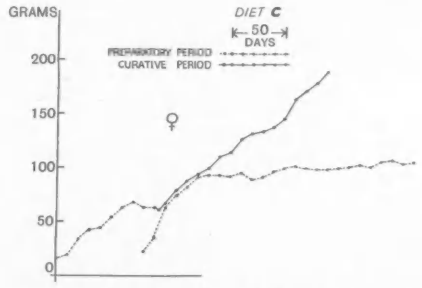

Fig. 2. Anomalous growth curve of negative control animal on vitamin A-free diet; cessation of growth with long survival (and no xerophthalmia).

considered "run out" when their growth has only slackened, without even actually ceasing, for a few days, and one may be permitted to wonder whether their "response" to vitamin supplements was wholly due to the supplement.

The onset of xerophthalmia in our stock is so uncertain that it has been found useless as a criterion of avitaminosis, though when it does occur it is invariably relieved or cured by administration of sufficient vitamin A, whether with or without resumption of growth. It is not uncommon, again, for "negative control" animals to reach a growth plateau, at 120, 150 or even 200 g. weight or more, and then to continue to live in apparently perfect health for weeks, or even months, with no change in diet or weight (see negative control animal,

Fig. 2). In so far as our experiments cover the same ground as those of Culhane [1932], we agree with her that the incidence of xerophthalmia does not appear to be markedly different with soluble caseinogenate and heat-treated caseinogen, and is both low and erratic with either. We have no experience of the alkaline-heated caseinogen that she found efficacious in the regular production of the eye-trouble.

It can, however, be said that the basal diet at present in use, combined with the technique of Nelson mentioned above, allows quite a large number of litters to respond satisfactorily to the diet, but the unexplained vagaries just described make it essential to discard whole litters that do not respond reasonably well to the diet, to compare as far as possible only isogenic animals and always to have a certain number of "negative controls" in each experiment.

A complete confirmation of our views both on the need for the use of isogenic animals and of the fundamental importance of an inbred stock is to be found in the fact that the variations of response to diet within a litter are far less than the variations between litters (see Series i below). This experience has been reported by workers in other branches of vitamin research [*cf.*, for example, Bourdillon *et al.*, 1931].

Experimental.

Preliminary series. These experiments will not be described in detail. Forty-five animals were distributed into seven groups receiving various diets of two main types, corresponding with diets A and B in Table II (except that no wheat germ was included in the diets of type B). The main source of protein was varied for the sub-types of both main types, heated caseinogen (Coward's "Glaxo casein"), the same caseinogen before heating, both fat-extracted and unextracted, a "soluble" sodium caseinogenate (Coward's "light white casein"), and the same after extraction of fat, being all examined. The results were inconclusive, but some reliance can be placed on the animals given the four diets:

(*a*) Type A with caseinogen.
(*b*) Type A with caseinogenate.
(*c*) Type B with caseinogen.
(*d*) Type B with caseinogenate.

The isogenic groups used each contained 3 males and 4 females (3 males and 3 females only in group (*a*)), and the animals were given the unsupplemented basal diets until they died or were moribund. The average survival periods were as follows:

(*a*) 131 days (2·5).
(*b*) 211 days (1·6).
(*c*) 156 days (1·7).
(*d*) 129 days (0·8).

The animals' condition at death varied from complete protection to acute xerophthalmia. On an arbitrary score of 3 = complete protection; 2 = slight xerophthalmia; 1 = severe xerophthalmia; 0 = acute xerophthalmia with blindness, the average condition of the animals can be calculated and is shown by the figures in brackets after the average survival period. The indication is that the caseinogenate protects rather less completely from xerophthalmia than does the heated caseinogen.

The other averages to be obtained from these figures are as follows:

Diet type A (13 animals)	...	...	...	174 days
Diet type B (14 animals)	...	...	...	143 ,,
Soluble caseinogenate (14 animals)	...	...		170 ,,
Heated caseinogen (13 animals)	...	...		144 ,,

There is thus an indication, though nothing more, first that diet B gives a more rapid "running-out" period than diet A, and subsequent experiments make it well-nigh certain that this is at any rate partly due to the lack in diet B of something present in fat-free wheat germ, and, secondly, that the soluble caseinogenate allows of longer survival than the heated caseinogen.

Series i. This had for its object the comparison of the three different types of diet shown in Table II. As it was wished to use 18 males and 18 females on each diet, the principle of isogenic control was abandoned in this case, and the animals were arranged as shown in Table I.

Table I. *Distribution of animals and diets used in Series* i (*see Figs. 3 and 4*).

	Litter-groups of 6	
Diets	Males	Females
A	i. *ex* 6 ♂ and 1 ♀ (30)	iv. *ex* 4 ♂ and 6 ♀ (36)
	ii. *ex* 6 ♂ and 3 ♀ (27)	v. *ex* 6 ♂ and 7 ♀ (30)
	iii. *ex* 6 ♂ and 7 ♀ (30)	vi. *ex* 2 ♂ and 7 ♀ (28)
B	i. *ex* 7 ♂ and 1 ♀ (28)	iv. *ex* 2 ♂ and 7 ♀ (28)
	ii. *ex* 7 ♂ and 1 ♀ (27)	v. *ex* 1 ♂ and 6 ♀ (26)
	iii. *ex* 6 ♂ and 1 ♀ (28)	vi. *ex* 3 ♂ and 6 ♀ (28)
C	i. *ex* 6 ♂ and 1 ♀ (30)	iv. *ex* 5 ♂ and 6 ♀ (31)
	ii. *ex* 6 ♂ and 2 ♀ (31)	v. *ex* 2 ♂ and 7 ♀ (32)
	iii. *ex* 7 ♂ and 3 ♀ (33)	vi. *ex* 3 ♂ and 7 ♀ (25)

Table II. *Percentage composition of diets.*

	Diet	A	B	C
Carbohydrate	Dextrinised rice starch	74	—	—
	Rice starch	—	55	50
Fat	Aerated cottonseed oil	—	10	—
	Arachis oil	—	—	10
Protein	Soluble caseinogenate	15	—	—
	Heated caseinogen	—	20	20
Salt mixture	Steenbock 40	4	—	—
	McCollum	—	5	5
Roughage	Agar-agar	2	—	—
Vitamin B	Marmite	—	5	5
	Dried yeast	5	—	—
	Wheat germ (fat-free)	—	1 g. fed separately to each animal per day	5
Vitamin C	Lemon juice	—	5	5
Vitamin D	—	Diet irradiated	20 units in 10 g. diet; irradiated ergosterol dissolved in oil of diet	100 units twice a week; irradiated ergosterol fed separately to each animal

Diet A is similar to that used by Coward, and the source of protein, described as "soluble caseinogenate," is identical with her "light white casein." Our diet, however, which was identical with an earlier diet described by Drummond,

Coward and Handy [1925] contained 3 % less yeast than Coward's. Diet B, without the supplement of wheat germ, had been used by us for some years; some preliminary experiments had shown that marked improvement in growth on this diet followed the daily administration of 1 g. of wheat germ that had been extracted with acetone to remove oil and fat-soluble vitamins. The addition may have brought about the improvement by virtue of its content of vitamin B, of extra protein, or of roughage, or of a combination of these. The replacement of the aerated cottonseed oil by refined arachis oil did not appear to introduce any traces of vitamin A, so that arachis oil was included in diet C, which is now the only vitamin A-free diet used regularly in our laboratories. In diets B and C the caseinogen used was the acid-precipitated heat-treated caseinogen described by Coward as "vitamin-free casein (Glaxo)." Diet A was fed dry and diets B and C as pastes. It will be seen that diets A and B differ in a number of respects, whereas diet C is simply an improved form of diet B, itself a modification of the diet at first advocated by Drummond and Watson [1923].

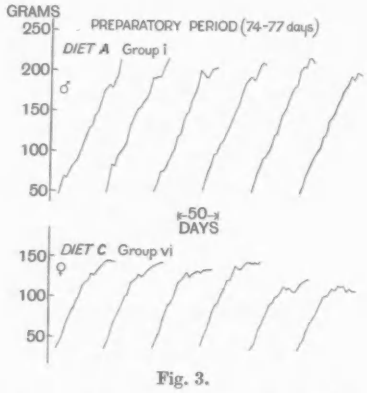

Fig. 3.

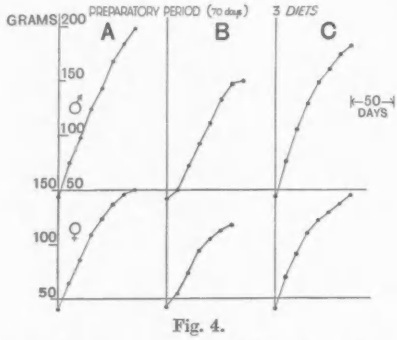

Fig. 4.

Fig. 3. Typical growth curves for two different (isogenic) groups on two different basal diets (see Table II).

Fig. 4. Average growth curves for groups of six animals each, on three different basal diets (see Table II) during "running-out" period.

In Table I we have included data as to the size of the original litter and the age (figures in brackets) of the litter groups at the beginning of the experiment; examination of all the average growth curves of the 18 groups showed that neither of these factors influenced significantly the animals' response to the diets. In Figs. 3 and 4 are given respectively the growth curves for all the animals of two typical groups (A i and C vi), and the average curves for all the males and all the females on each diet. The behaviour of the animals in this experiment showed

(1) a very reasonable regularity of response of the individual animals in the same litter groups;

(2) a fair regularity of average response of the groups of one sex on the same diet;

(3) the usual superiority of male growth rate over female;

(4) a considerable difference of average response to the three different diets.

How far the difference noted under (4) is a true expression of different dietary conditions, and not of differences between the litters, selected at random, for the three diets, is uncertain, but in so far as it is so, diets A and C would seem to approximate more closely to a theoretically sound diet than does diet B. The experiment was, however, concluded after 70 days, and it became necessary to probe rather more deeply into the possible causes of the difference in average response to the three diets. The difference between response to diets B and C is, as we have mentioned, not attributable to the difference in the oils used, unless the peroxide in the aerated cottonseed oil was causing destruction of the vitamin D dissolved in it. The evidence of other preliminary tests, however, suggests that this was not the case, but that the wheat germ supplement was almost solely responsible for the animals' improved growth.

Direct comparison between the fat-free diet A and diet C is less simple, since two different sources of protein were used and, according to Coward, this affects the response of the animals to curative treatment rather than to the deprivation of vitamin A. We have therefore investigated the effect on response to cure of animals on diets of both types A and C, using each source of protein with each diet.

Series ii. Forty-eight animals were used in this test, six groups, each of four isogenic males, and six groups, each of four isogenic females. One animal of each group received a diet of type A containing one of four sources of protein, three being different samples of heated caseinogen and one a sample of soluble caseinogenate. The distribution of animals and diets is shown in Table III. Two groups of the males and two groups of the females were given no curative treatment; all the other animals received after the "running-out" period a daily supplement of one drop (20–25 mg.) of cod-liver oil known to be a good source of vitamin A (Carr-Price blue value about 12). All the latter animals remained alive, and in two cases (females) in which xerophthalmia had developed

Table III. *Distribution of animals and diets used in Series* ii (*see Figs. 5 and 6*).

Diet type A.

Litter-group	Caseinogen 1	Caseinogen 2	Caseinogen 3	Caseinogenate
♀ I, II, III, IV	+	+	+	+
V, VI	0	0	0	0
♂ VII, VIII, IX, VI	+	+	+	+
X, XI	0	0	0	0

+ = Received cod-liver oil supplement. 0 = Received no supplement.

this was cured. All the unsupplemented males died or were moribund after 150 days or less on the diet; five of the unsupplemented females died in 120 days or less, while the other three, though still alive after 175 days, had made no growth for some weeks. Of these 16 negative controls one male and one female developed slight and one female definite xerophthalmia.

In Figs. 5 and 6 are shown the average growths on each diet of the eight animals (four males and four females) which received the supplementary dose of cod-liver oil; the 16 negative control animals are omitted from these averages, since three died before the period of supplementary feeding began, and since clearly none of the other 13 can be included with the animals whose response to cure is being discussed. Examination of the individual groups showed that response to cure followed litter-grouping and not the nature of the source of

protein used, and the latter point is made clear by the average curves of response. Thus the animals in male groups VIII and IX made good response; those in male group VI and in female groups III and IV made fair response; those in male group VII and in female groups I and II made very poor response; male groups X and XI and female groups V and VI were negative control groups. It is perhaps noteworthy that three of the four negative control females in

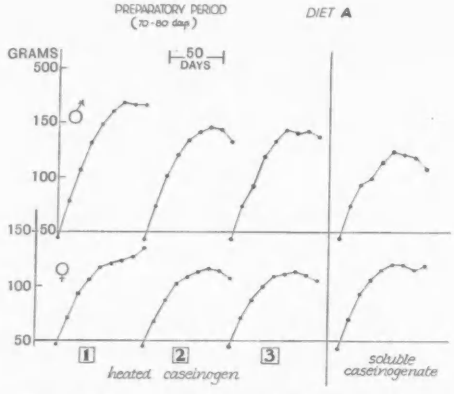

Fig. 5. Average growth curves for groups of four animals each, on basal diet A (see Table II), using two different types (3 and 1 samples) of protein, during "running-out" period.

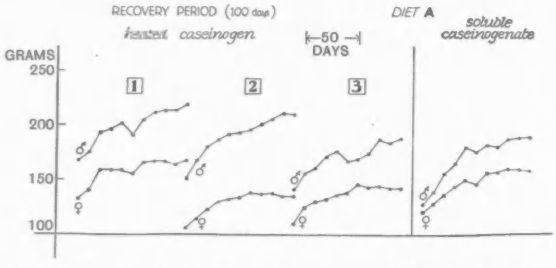

Fig. 6. Average growth curves for recovery period, corresponding with preparatory period illustrated in Fig. 5.

group VI survived the experimental period, and that the males in group VI, which were part of the same litter, made only fair growth response to curative treatment, so that animals having, theoretically, excessive "reserves" nevertheless failed to make good response to curative treatment.

Each average curve for the "running-out" period is for four animals up to 70 days and for two animals for the last 10-day periods, since 2 male and 2 female groups were put on to supplementary doses between the 70th and 80th days of experiment. The average curves for the curative period are, however, all constructed with the first day of supplementary feeding as day 0, and represent a curative period of 100 days.

Inspection of the curves reveals the following characteristics.

1. A slightly poorer growth performance during the preparatory period by the males receiving caseinogenate than by those receiving caseinogen.

2. No significant difference in the effects of the two proteins in the "running-out" period of the females.

3. A slightly more vigorous growth by both sexes on the first of the three heated caseinogens than on the other two.

4. No significant difference in response to cure associated with the two different caseinogens. The slightly greater initial growth of the males receiving the caseinogenate is most probably associated with their lower average weight at the beginning of the curative period.

5. A very poor total response to curative treatment, compared with that reported by many other investigators, including especially Coward. All the growth-curves show a tendency to "flattening" at about the same weight, 200 g. for males, 150 g. for females.

Series iii. In this experiment only two litters were used, one of 8 males and one of 6 males and 4 females. All these animals received diet C, but 4 males from the first litter and 3 males and 2 females from the second had only heated caseinogen (sample No. 3) as a source of protein, while the rest had the soluble caseinogenate only. Two males on each source of protein in the first litter and 1 male and 1 female on each source of protein in the second litter received no supplement and acted as negative controls, while all the other 10 animals received from the 94th to the 158th day daily doses of cod-liver oil (20–25 mg. of an oil with a Carr-Price blue value of about 10). Owing to the relatively small number of animals, the averages have been calculated for males and females together, and the results are plotted in Fig. 7.

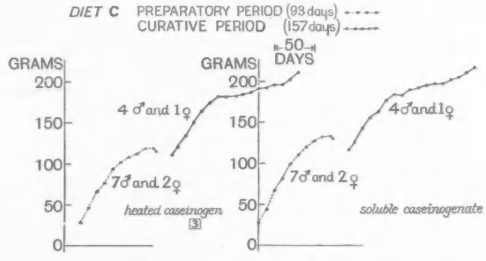

Fig. 7. Average growth curves for nine animals (preparatory period) and five animals (recovery period) on basal diet C (see Table II), using two different types of protein.

Owing to the number of animals used it is undesirable to stress even the negative results of this experiment, which shows no significant difference in effect on running-out weight, running-out time or extent of recovery between the two sources of protein. The similarity of these average growth-rates is closely reflected in the individual curves for those animals receiving curative treatment; two of the males receiving no supplement, however, grew slowly but steadily throughout the experimental period, at the end of which they were comparable in weight with isogenic animals which had run-out and then been given cod-liver oil. Both of these animals came from the group fed on caseinogenate and are responsible for the slightly steeper average curve for the running-out period of these animals; but for them the two sets of curves would have been well-nigh

indistinguishable. The average growth increase of the animals receiving heated caseinogen for the first 37 days of the recovery period was 54 g.; for the animals receiving soluble caseinogenate the corresponding figure was 60 g. Here again we have no significant difference in response and a markedly sub-normal growth rate for both groups of animals during the recovery period.

These results raised at once the question whether diet C, though it seemed to possess no theoretical disadvantages, was indeed the ideal vitamin A-free diet for which we were seeking. This could be tested only in one way, and the test was made in the next experiment.

Series iv. Here six isogenic groups of males were used; the five males of each group were distributed over the five diets described below.

Group G. Kitchen-scrap supplemented diet, as used for "genealogical" stock.

Group S. Stock diet as used for breeding stock.

Group P. Diet C, containing heated caseinogen and supplemented with adequate vitamin A[1].

Group K. Diet C containing heated caseinogen, unsupplemented for 90 days, and then supplemented as for Group *P*.

Group C. Diet C containing heated caseinogen, unsupplemented.

The experiment was continued for one year, with the result summarised in Fig. 8. The curve for group *G* has been omitted since it ran continuously parallel

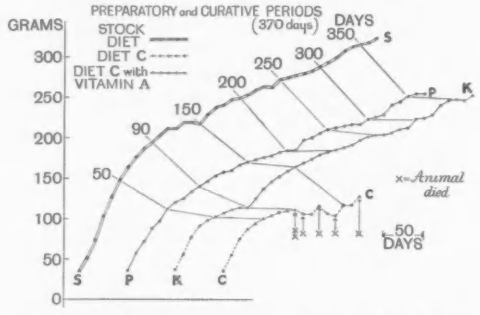

Fig. 8. Average growth curves for four groups of six isogenic animals on stock, supplemented basal, and unsupplemented basal vitamin A-free diets, for whole experimental period of 370 days.

with that for group *S*; at the end of 370 days the average weight of the animals in group *G* was 344 g., and of those in group *S* 323 g., and the spread of the individual weights suggests that the difference is not significant. The conclusions to be drawn from the four curves shown do not allow of any doubt.

(1) Diet C, supplemented with adequate vitamin A, is not capable of giving normal growth.

(2) Diet C, unsupplemented, allows animals to "run out" in such a manner that, when adequate vitamin A supplement is added to it, the animals will make as much recovery as the limitations of the diet permit. The average final weights of the animals in groups *S*, *P* and *K* were: *S*, 323 g.; *P*, 254 g.; *K*, 252 g.

(3) Diet C, unsupplemented, is incapable of maintaining growth beyond a point definitely below that reached if it is supplemented with vitamin A, either prophylactically or curatively.

[1] Unsaponifiable matter from 50 mg. of cod-liver oil having a Carr-Price value of 10, determined on the oil, and 16 determined on the unsaponifiable matter.

Discussion.

The results, in their bearing on the source of protein in vitamin A-free diets, do not confirm those reported by Coward, and no explanation of the discrepancy is put forward. Her standard vitamin A-free diet, it is true, contains 8 % of an active dried yeast, and it is just possible, though in my opinion very improbable, that this is a more complete source of the B-vitamins than the 5 % of marmite and 5 % of wheat germ present in diet C. This point is being tested in further experiments now in hand[1]. It is also a fact that several of our batches of wheat germ were extracted with acetone; the first runnings of extract contain considerable amounts of water, since wheat germ has a moisture content of 12–15 %, and there is the possibility that the aqueous acetone may be extracting some of the vitamin B from the germ. This point is also under investigation.

I have had the benefit of Dr Coward's own views on these results, and we are unable even jointly to offer any explanation. The beneficial effect of wheat germ on my diet B is consistent with Dr Coward's finding that wheat germ is a source of the unknown "factor" when fed at a level of at least 20 %. Diet C, however, only contains 5 % of wheat germ; it should therefore be inadequate as a supplement of her factor to the heated caseinogen, while the soluble caseinogenate should need no such supplement. Yet the experiments in Series iii detected no difference whatever between the two sources of protein in a diet containing 5 % of wheat germ. Again, there can be little question as to the adequacy of the vitamin D supplies to animals receiving diet C. They were given about 200 units per week; Coward (private communication) gives her animals 8 units, certainly more than sufficient. The poor recovery of the animals on so high a dose as the equivalent of 50 mg. of good cod-liver oil is doubtless a summation of two (or more) causes—the relatively high weight at which the animals "run out" and the unknown limiting factors of the basal diet itself.

A possible explanation of some of our results might be given in terms of vitamin A storage by the animals during their pre-experimental (including prenatal) lives. This should result in an unduly long period of running out, with the attainment of an unduly high growth plateau, so that the animals would have a potentially lower recovery rate than animals which stopped growing sooner. There are, it seems to me, two unanswerable objections to this suggested explanation. First it can in no way account for the failure of the animals in group *P*, Series iv, to make growth equal to that of the animals in groups *G* and *S*, since the three groups were absolutely comparable in pre-experimental history. High initial vitamin A reserves can hardly explain failure to reach normal weight with adequate vitamin A administration. Secondly, we have experimental evidence which is extremely difficult to reconcile with the current conception of vitamin A storage and its effect on resistance to the ill-effects of deficient diets. (See the following paper, p. 17.)

We are therefore compelled to accept the simple explanation that the failure of animals receiving vitamin A and diet C to make growth approximating to that of stock-diet fed animals must be associated with some unrecognised deficiency of diet C. Possible sources of such deficiency are:

i. Shortage of one or more substances in a diet qualitatively correct, *e.g.* vitamins of the B group, amino-acids.

ii. Absence of some constituent or factor already known, *e.g.* vitamin E, roughage.

[1] So far they quite refute the idea that diet C is in any way deficient in vitamin B.

iii. Absence of some new or unrecognised factor, *e.g.* "physin" [*cf.* Mapson, 1932], a caseinogen factor [*cf.* Coward *et al.*, 1929, 1, 2].

iv. A combination of two or more of these causes.

Experiments are in hand, and more are planned, to investigate some of these possibilities, but they must of necessity be of fairly long duration, since they involve observing the weight and condition of adult animals reared from youth on experimental diets.

Meanwhile the apparently inherent limitations of our basal diets lead us to emphasise the desirability of assessing the growth-producing potentialities of it or any similar diet, wherever possible, by carrying out positive control tests of the prophylactic as well as of the curative type.

Summary.

1. Attention is drawn to various types of anomaly shown by rats fed upon vitamin A-free diets, both during the preparatory "running-out" period and under curative treatment.

2. With two different vitamin A-free diets, one containing no fat, running-out time, weight and degree of recovery under curative treatment seemed to be independent of the sources of protein examined—a heat-treated "vitamin-free" acid caseinogen, and a "soluble" sodium caseinogenate ("light white casein").

3. In no case was growth under curative treatment resumed to an extent equal to that of normal animals receiving stock diets.

4. Evidence is submitted that the vitamin A-free diets used must have been deficient in more than vitamin A.

5. The bearing of these results on the technique of vitamin A testing is discussed.

I wish to take this opportunity of acknowledging invaluable assistance in this work from Miss E. Allchorne who had general charge of the animals, and from Miss P. Cook who assisted with the feeding and weighing.

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III. INVESTIGATIONS INTO VITAMIN A-FREE BASAL DIETS. II.

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(*Received December 1st, 1932.*)

The experiments described in this paper were carried out with a two-fold object. It was desired first to discover what effect deprivation of vitamin A had on the lymphatic tissue in various parts of the body, and secondly to observe simultaneously whether the difference in growth-promoting properties of a complete diet and of a vitamin A-free diet supplemented with vitamin A, already described in a previous communication [Bacharach, 1933], could be confirmed and correlated with the reserves of vitamin A in the livers of the experimental animals. The results of the observations on the lymphatic tissues will not be reported here in full (see note on p. 21).

Eighteen animals, nine of each sex, were used in this experiment, and their arrangement is shown in Table I. One group of six animals was given the full stock diet (S) described in the earlier paper (p. 6). This diet was also the diet upon which the parents of all the experimental animals had been fed during the whole of their adolescence and maturity, and upon which the experimental animals in groups 2 and 3 had been fed up to beginning the feeding of the vitamin A-free diet.

The second group was given the vitamin A-free diet (C) described in the same paper (p. 14). To this diet was added a daily dose of the unsaponifiable matter from fish-liver oil dissolved in arachis oil; the amount of this dose and the method of expressing it are discussed below. No irradiated ergosterol was fed to these animals.

The third group of animals received diet C, but no supplementary source of vitamin A.

The experiment was continued for 150 days, at the end of which all the animals were killed. Their livers were tested for vitamin A in the manner described below, and various other portions of tissues and organs were set on one side for histological examination (see note on p. 21).

In the absence of any unit of vitamin A as yet in universal use (the international standard solution of carotene having not been long enough in circulation for the necessary biological determinations to be made) it is proposed to express the activity of the concentrate fed to the animals, and the vitamin content of their livers, in terms of a standard cod-liver oil, compared colorimetrically by the antimony trichloride test. The oil adopted as a standard has a Carr-Price value of (approximately) 10, and a value of 16 when this is determined by the method of Smith and Hazley [1930]. The latter value has been taken for purposes of comparison in this paper. All figures for extract and livers given below are in terms of equivalent milligrams of such an oil.

It may be added that such an oil has a content of 10β per g., in the unit of Smith and Hazley, gives 900 B.U. per g., calculated by the method of Moore [1930] and has a value of $E^{1\%}_{1\text{ cm.}}\,328m\mu = 0{\cdot}7$.

The vitamin A content of the extract fed to the animals in group 2 was determined in the manner described by Smith and Hazley [1930] and was that of 50 mg., of the defined cod-liver oil. The livers were treated with dilute alkali and the extract for the antimony trichloride test was made exactly as described by Moore [1930].

Fig. 1 shows the average growth curves of the animals in all three groups. The three litters were respectively 20, 21 and 24 days old at the time of the beginning of the experiment, and there is little doubt that the relatively high weight at which the animals in group 3 showed signs of ceasing to grow, and the relatively long time that it took them to reach a growth plateau, are associated with the length of time elapsing before they were put on to the deficient diet [*cf.* Nelson, 1928].

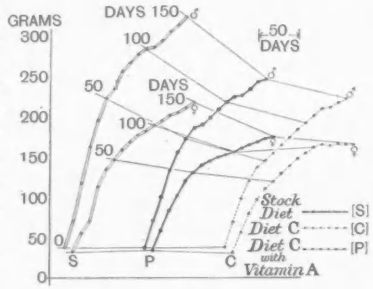

Fig. 1. Average growth curves for three groups of three males and three groups of three females on stock, supplemented basal, and unsupplemented basal vitamin A-free diets for experimental period of 150 days.

Although, for obvious reasons, no direct measure was possible of the vitamin A reserves of the animals at the beginning of the experiment, an approximate measure was obtained by taking 12 young animals entirely at random from the general stock of which the experimental animals had formed part, and carrying out a colorimetric determination of the vitamin A content of their pooled livers. This proved to be 100 mg. C.L.O. per liver. The animals all weighed approximately 40 g. and were between 4 and 5 weeks old. They were weaned and had been eating diet S for themselves for a week or so. It is clear that the experimental animals began to receive the three diets at a time when their vitamin A reserves, at any rate in the liver, were definitely low compared with the undoubtedly high reserves of vitamin A that must have been present in the livers of their mothers, as evidenced by the livers of those experimental animals, described in Table I, which received the stock diet S throughout the experimental period.

The young experimental animals used in this investigation had received, directly or indirectly, diet S from birth until they were about 4 weeks old;

Table I. *Final weights and vitamin A liver reserves of 18 rats on three diets.*

		Diet S (stock)		Diet C + vitamin A		Diet C	
Litter	Sex	A	M	A	M	A	M
2561	♂	4,400	279	1100	202	0	177
	♀	19,000	190	1250	154	0	150
2574	♂	27,500	367	1750	260	0	225
	♂	33,000	334	1300	280	c. 100	270
	♀	26,000	248	1550	178	c. 200	170
2566	♀	14,000	230	c. 350	198	0	180
Averages	♂	16,200	327	1400	247	30	224
	♀	16,500	223	1050	177	70	133
	Both sexes	16,400	275	1250	212	50	195

M = Final body-weight of animal.
A = Vitamin A content (in mg. of "standard" oil) of animal's liver.

when they were suckling, their mothers must have been thoroughly well supplied with vitamin A. The relatively low reserves of the newly weaned animals constitute, therefore, a further illustration of a phenomenon to which Dann [1932] and others have called attention and seem to afford further support for their suggestion that the passage of vitamin A from mother to suckling is limited by the extent to which it can pass from the maternal organism into her milk supply. Dann has found (private communication) that the liver reserves of the young animals are still lower if they have no access to supplementary sources of vitamin A (liver, green leaves, *etc.*) after suckling.

Table I shows the final body-weight and the vitamin A content of the liver of each of the 18 experimental animals, as well as the average figures for body-weight and vitamin A content.

Discussion.

These results afford further confirmation of some deficiency in the vitamin A-free diet, other than the absence of vitamin A, to which attention has already been called [Bacharach, 1933]. The ingestion of 50 mg. (C.L.O.) of vitamin A per day for 150 days certainly enabled the experimental animals to remain in positive vitamin A balance, since their liver reserves were considerably higher at the end of the experimental period than at the beginning, yet their growth was only slightly better than that of animals receiving the same basal diet but no supplementary source of vitamin A, and very markedly inferior to that of animals receiving a full stock diet. However, the animals which had received the full stock diet accumulated a far larger reserve of vitamin A than those which had received the supplemented vitamin A-free diet. A number of possible explanations of these phenomena could obviously be brought forward, but there is no experimental evidence either in favour or against any one or other of them. The animals receiving the full stock diet S were obtaining their vitamin A partly from the dried milk, from which they were presumably getting about 1 g. of butter-fat per day, and from the supplementary supply of liver; they were also receiving carotene, from the supplementary supplies of green leaves and possibly partly from the butter-fat. It is not, therefore, possible to say how much vitamin A, actual and potential, they were receiving per day; their liver reserves were about ten times as high as those of the animals in group 2 but it seems highly improbable that they were obtaining over ten times as much vitamin A. For the animals in group 2 we know with some exactness how much vitamin A was being consumed; at a level of 50 mg. per day they must have received a total supply of 7500 mg. Of this only 1/6th was found in the livers at the end of the experiment; some 85 %, therefore, of the vitamin A fed to these animals was either lost from the body unchanged in the faeces, or destroyed during its passage from the alimentary canal to the place of storage in the liver. In spite of the fact, therefore, that the animals were receiving a diet containing 10 % of fat, which is believed to facilitate the absorption of fat-soluble vitamins, they did not appear to have stored much more than 15 % of the vitamin A ingested.

For the animals in group 3 the average vitamin A reserve was half that found for the control group of 12 young animals referred to above, but the difference between the 50 and 100 mg. respectively found in the livers of the two groups was barely outside the experimental error of the colorimetric estimations made by us.

It is clear that the animals in group 1 are adequately supplied with vitamin A and all other dietary requirements, and that the animals in group 3 in spite of

their relatively low vitamin A reserve at the beginning of the experiment, and in spite of the fact that this reserve showed little change at the end of the experimental period, were able to continue on a vitamin A-free diet for a considerable period. The animals in group 2 occupied an intermediate position. They were able to accumulate an appreciable amount of vitamin A in their livers and to make somewhat better growth than the animals in group 3, and they would doubtless have continued growing more or less indefinitely, in the manner previously found for animals similarly fed [Bacharach, 1933]. Investigations now in progress have for their object a further attempt to improve diet C so as to secure that animals receiving it and a supplement of vitamin A shall show normal growth and, indeed, reproduction also, if this can be achieved, and also to secure that animals receiving diet C from an early age shall cease to grow sooner, and at a lower weight, than do most of the animals at present receiving it. Evidence is already to hand that placing the young suckling animals, with their mothers, on the basal diet at 9 or 10 days of age brings about a very great improvement in this second respect, and therefore raises still further queries as to the soundness of the present assumptions about the passage of vitamin A from the maternal to the embryonic and suckling organism, and the effect of vitamin A reserves in the young animals on their growth potentialities. Either some hitherto unsuspected phenomenon must be responsible for the relation between "running out" time and pre-weaning diet, or a vitamin A reserve of 100 mg. (C.L.O.) in the weaning rat is a significant factor in protecting it against vitamin A deficiency.

Summary.

1. Three comparable groups of six animals were fed upon a stock diet, a vitamin A-supplemented vitamin A-free basal diet, and the basal diet, respectively.

2. In conformity with earlier findings, the animals in the first group grew more rapidly and reached a greater final weight, at the end of 150 days, than those in the second group. The animals in the third group had, in the same time, reached an inconveniently high weight, and barely ceased to grow.

3. The liver reserves of vitamin A in each animal were estimated by Moore's method. The values were consistent with the growth performance in the three groups, and were compared with those of young control animals.

4. The bearing of these findings is discussed, with special reference to problems of technique in vitamin A testing and to the relation between liver reserves of vitamin A in adult does and in their young and the subsequent growth performance of the offspring, when these are given a vitamin A-free diet.

Acknowledgment is again gladly made of invaluable assistance from Miss E. Allchorne and Miss P. Cook in the carrying out of the experiments.

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Note added February 1st, 1933. Since this paper was written, we have heard from Dr V. Klemola that he has concluded the histological examination of all 18 rats, and that the results will in due course be published, along with subsequent findings carried out with further experimental material. Here it must suffice to quote his summary. "The histological changes between animals receiving diets S and C (unsupplemented) were marked. The latter showed a diffused distribution of lymphocytes in the follicular patches, whereas the former showed the normal compact structure, with clearly differentiated central "reaction centres." The hyperkeratinisation shown by the epithelial layers of skin, fore-stomach, *etc.*, was typical of the pathology of deficiency, as also was the diminution in hair growth, the reduction of length and thickness of fibre being signs of involution. The animals receiving the supplemented diet C showed normal characteristics, except in two cases where the lymphatic reticular structure was not quite normal in appearance, though that departure from normality may quite possibly be due to *post-mortem* changes." These findings support the view that the animals receiving unsupplemented diet C were indeed in a state of definite vitamin A hypovitaminosis, and that the failure of the animals receiving supplemented diet C to show as good growth performance as the animals receiving diet S was associated with some deficiency other than vitamin A shortage.

IV. THE CALCIUM CONTENT OF MUSCLE.

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(*Received November 21st, 1932.*)

The facts that even excised muscle is sensitive to lack of calcium and that various forms of muscle tremors have been associated with low serum-calcium have led to the idea that changes may occur in muscle-calcium in various forms of tetany. The marked influence of diet on bone- and blood-calcium has suggested that muscle-calcium might also be similarly affected by this factor. Previous analyses of muscles have indicated a fairly wide range in the calcium content of normal muscle without showing any marked deviation from this average range under special conditions. Heubner and Rona [1923] failed to find any change in the calcium of dog muscle when the dogs received large doses of calcium chloride subcutaneously. Dennis and Corley [1925] failed to influence the calcium content of rabbit muscle by giving large doses of calcium lactate or chloride orally, with or without treatment by ultra-violet rays. Loughridge [1926] reported a drop in the muscle-calcium of dogs after parathyroidectomy. Dixon, Davenport and Ranson [1929] found on the other hand that the muscle-calcium of a series of parathyroidectomised dogs fell within the same range as the muscle-calcium of a series of control animals. Haury [1930] found that the average calcium of the muscle of a batch of rachitic rats was lower by 40 % than that of a batch of control animals given a similar diet to which phosphate had been added in sufficient quantity to prevent the development of rickets. Bülbring [1931] gave rats diets containing 0·86, 0·46 and 0·03 % calcium. The muscle-calcium of rats on diets poor in calcium did not differ from that of those receiving 0·4 % calcium, but the rats receiving the diet rich in calcium (0·8 %), which contained calcium lactate in addition to the ordinary salt mixture, showed a muscle-calcium generally higher than that of their litter-mates. On the two diets poorer in calcium, parathyroidectomy and parathormone injections produced no demonstrable consistent changes in muscle-calcium, but on the diet rich in calcium, muscles from parathyroidectomised animals or from animals receiving parathormone injections were richer in calcium than those of litter-mates on the other diets. The figures shown by all these workers seem to indicate that normal muscle-calcium may vary from about 4 to 25 mg. per 100 g. of fresh tissue.

Since, however, calcium metabolism is known to be largely influenced by the dietary conditions of the stock from which animals are taken, series of estimations of muscle-calcium were made on control rats and also on their litter-mates kept under varying conditions. It was hoped in this way to demonstrate small changes which might be concealed by large differences among the controls taken at random from unknown stocks, as in most of the experiments referred to.

Experimental.

Wistar Institute rats from the Glaxo laboratories were used, and the animals were kept in cages in groups of three or four and were fed their diets *ad lib.* Those receiving special doses of food were removed individually from the cages for this purpose. As far as possible, litters were equally distributed through the experimental groups, and all experimental animals were always compared with their litter-mate controls in interpreting the results. The animals were killed by bleeding from the neck.

The analyses were carried out (except where otherwise stated) on the thigh muscles, which were dried in silica crucibles and then ignited and extracted with 5 % HCl. The filtrate from this extraction was made alkaline to methyl red with ammonia and then just acid with glacial acetic acid, and the calcium was precipitated as oxalate, using large excess of oxalate for the purpose. At least 24 hours had to be allowed for the complete precipitation of the calcium oxalate. The muscle ash was frequently found to be contaminated with silica from the crucibles, and repeated extraction of the ash was necessary to remove all the calcium. This was a potential source of serious error. A large number of control analyses were however carried out, which indicated that the analyses were accurate to ± 5 %, except in the case of the rachitic or other small animals, where only small amounts of muscle were available for analysis, so that the final titration figure was so low that errors might amount to a maximum of ± 10 %. The results were all interpreted with these technical limitations in mind[1].

The distribution of calcium in the muscles of the two sides of the same animal was first studied (Table I). For this purpose the muscles of the legs and strips

Table I.

Calcium in mg. per 100 g. of fresh muscle.

	Rat ...	1	2	3	4	5	6	7	8	9	10	Av.
Hind-legs	Right	6·0	6·4	7·0	6·5	6·2	6·0	7·5	5·5	6·0	6·8	6·4
	Left	5·7	6·5	8·5	5·6	5·8	7·3	8·8	8·1	8·5	5·9	7·0
Fore-legs	Right	9·0	10·1	9·2	—	9·4	11·4	13·9	—	10·5	8·0	10·2
	Left	8·5	10·3	18·6	—	8·6	9·0	16·5	—	8·3	7·7	10·9
Abdominal and pectoral muscles	Right	6·4	5·9	8·4	14·0	7·2	6·6	(10·3)*	9·2	8·1	8·0	8·2
	Left	6·4	7·7	6·4	7·2	7·3	7·1	—	9·0	11·3	8·4	7·9

* Muscle of two sides combined.

from abdominal and pectoral muscles on each side of the medial line were used. The analyses were carried out on muscles from 10 animals on stock diet S (see p. 24). Of the 27 pairs of muscles so examined, 12 varied from their mean by less than ± 5 %, 18 by less than ± 10 %, and 25 by less than ± 15 %. In two pairs, muscle from one side had a calcium content twice that of the muscle from the other side. These analyses were all carried out on small samples of chopped muscle, as the rest of the muscle was required for another investigation; under these conditions an error of ± 10 % might be regarded as the limit of accuracy for the estimation as carried out.

[1] The use of platinum crucibles facilitated the acid extraction of the ash and increased the accuracy of the analysis, but only one such crucible was available. This was used in each set of experiments, but the results so obtained did not differ consistently from those derived from the analyses in silica.

The influence of the calcium phosphate and vitamin D contents of the diet was studied in two experiments. For the first the following diets were used.

(1) Diet R. Steenbock and Black's diet 2965 [Steenbock and Black, 1925]; Ca 1·3 %, P 0·45 %, Ca/P = 3·0.

(2) Diet P, *i.e.* diet R + 3·5 % KH_2PO_4; Ca 1·3 %, P 1·2 %, Ca/P = 1·1.

(3) Diet R + E, *i.e.* diet R + daily doses of ergosterol.

(4) Diet P + E, *i.e.* diet P + daily doses of ergosterol.

(5) Diet S. Steenbock's stock diet + 15 % dried milk [Bethke, Steenbock and Nelson, 1923]; Ca 0·37 %, P 0·48 %, Ca/P = 0·73.

(6) Diet S + carbonate. S + 1·5 % $CaCO_3$; Ca 1·1 %, P 0·51 %, Ca/P = 2·1.

(7) Diet S + phosphate (S + 2 % calcium phosphate); Ca 1·08 %, P 0·92 %, Ca/P = 1·1.

Forty rats were divided between the first four groups, each group containing 5 males and 5 females. The animals were put on to the diets at the age of 25–28 days. The females were killed after 36 days and the males after 42 days on the diets.

Fifteen other rats left from the same litters were divided as evenly as possible as regards litters into three groups and were put at the age of 30 days on to diets S, S + carbonate, and S + phosphate, on which they were kept for 49 days. The calcium contents of the muscles of all these groups are shown in Table II. To demonstrate the influence of these diets on general calcium metabolism, the average calcium content for the femora + tibiae of the rats in each group, and the serum-calcium as estimated on serum from the mixed bloods of all the rats in each group are also given.

Table II.

Muscle-Ca in mg. per 100 g. fresh tissue.

♂ Diet ...	R+E	P+E	R	P	S	S+carbonate	S+phosphate
Muscle-Ca Max.	6·7	8·0	8·1	8·1	—	—	—
Min.	6·0	6·6	6·5	5·6	—	—	—
Av.	**6·3**	**7·4**	**7·2**	**7·2**	**6·1**	**5·7**	**5·4**
Standard deviation σ	0·30	0·60	0·58	1·0	—	—	—
Serum-Ca (mg./100 cc.)	12·2	11·8	10·3	8·6	—	—	—
Bone-Ca (% on fresh bone)	10·5	11·5	7·4	10·4	12·8	12·9	14·1
♀							
Muscle-Ca Max.	18·2	8·1	8·2	23·2	8·7	8·6	6·1
Min.	6·2	6·2	6·3	6·6	5·2	5·2	5·1
Av.	**8·9**	**7·2**	**7·2**	**11·1**	**6·5**	**6·3**	**5·5**
σ	6·5* 2·4 0·4*	0·9	0·95	6·2* 7·6 1·4*	1·4	1·4	0·44
Serum-Ca (mg./100 cc.)	11·4	11·0	10·3	8·7	10·7†	10·8†	10·4†
Bone-Ca (% on fresh bone)	10·6	11·9	8·4	10·6	13·1	13·9	14·6

* Figures derived by excluding the one exceptionally high figure in the group.
† The 1 ♂ included in this group.

With the exception of one female on diet R + E, with a muscle-calcium of 18·2, and two females on diet P with muscle-calcium contents of 13·6 and 23·2, all the figures range from 5·2 to 8·7, and no significant difference is demonstrated between the sexes on the same diet, nor between animals on different diets, nor between animals aged 8 weeks (♀ on R, R + E, P and P + E) and animals from the same litter aged 11 weeks (♀ on S, S + carb. and S + phosphate). Comparison of litter-mates also fails to show any consistent influence due to the diets. This is in marked contradistinction to the influence of the diets on

bone-calcium, which is adequately indicated by the average percentages of bone-calcium, since only one of the 10 rats on diet R had a bone-calcium which was not markedly less than that of all its litter-mates on other diets, while of the 10 rats on diet P + E only one failed to exceed the bone-calcium of its litter-mates on diets R + E and P. The serum-calcium content as estimated on the pooled blood samples from the groups also seems to suggest an influence of the diet on serum-calcium.

These results fail to confirm Haury's finding that the muscle of rachitic rats contains less calcium than that of non-rachitic animals. This is equally true whether the comparison is made with a diet closely similar to the rachitic diet but containing vitamin D (R + E), or containing enough phosphorus to give a Ca/P ratio of 1 (diet P), or with both these anti-rachitic conditions present (P + E), or on the other hand with a complete diet adequate in vitamins (S), or with the modifications which give a high Ca/P ratio or a normal Ca/P ratio with high calcium and phosphorus.

The effect of vitamin D in ample quantity but not in excess was further studied by giving young rats the stock diet S and adding to the diet of one of each litter-mate pair a daily dose of irradiated ergosterol equal to ten times the quantity just necessary to give the line test in a rachitic animal in 3 days. This experiment was carried on for a period of 12 weeks, but was complicated by the fact that at the end of 8 weeks two animals died of pneumonia. The rest of the group showed loss of appetite for a day or two but rapidly became normal in appearance. Out of a total of 40 rats, however, only 7 continued to grow at a rate which could be regarded as normal, and at the end of 12 weeks they were all killed. At autopsy, one or two showed actual pus in the lungs, and most showed scarred lungs and calcareous deposits in the liver. Table III shows, however, that the muscle-calcium was unaffected by either the disease or the irradiated ergosterol in the quantity given. The number of litter-mate pairs which differ from their mean by less than ± 10 % is 68 % of the total, and all the pairs differ from their mean by less than ± 20 %.

Table III.

Muscle-Ca in mg. per 100 g. fresh tissue.

	No ergosterol	Ergosterol	$CaCl_2$ added
No. of animals ...	16	12	10
Max.	7·7	7·8	8·5
Min.	5·9	5·6	5·6
Av.	**6·6**	**6·5**	**6·4**
σ	0·55	0·71	0·87

Table III also shows the muscle-calcium content of rats which each received 1 cc. of 10 % $CaCl_2$ solution mixed with their daily diet (which was the ordinary stock diet S). All the diet was not always consumed, but the animals grew well, indicating that they were taking an adequate amount. In this way they received a varying quantity of additional calcium daily in a soluble form. Only 10 rats were available at the time, and no litter-mates were kept on the ordinary diet, so that strictly the group cannot be compared with the others in Table VI. The results are however so similar to the others found for this breed of rat in all these experiments that it was considered justifiable to include them here.

The muscle-calcium of animals in which a state of hyperexcitability or active tetany had been induced by various means was next investigated. The methods used were (*a*) the injection of guanidine salts, (*b*) the feeding of phosphates or

irradiated ergosterol to rachitic animals, (*c*) parathyroidectomy. Muscles were examined from animals in varying stages of hyperexcitability. Batches of rats on stock diet S were given non-lethal doses of guanidine salts on alternate days. (Most rats tolerated a dose of 200 mg. of guanidine per kg. of body weight, given intraperitoneally (I.P.) as lactate of p_H 7·0.) On the days on which no guanidine was injected, the rats reacted similarly to animals in latent tetany and were slightly more excitable than their control mates. Guanidine group A (Table IV), shows the muscle-calcium values for these rats. In order to eliminate the effects of mere injection, when the experimental animals received guanidine, the controls received injections of equal quantities of normal saline, or of ammonium or of sodium salt solutions prepared so that the ammonium or sodium was molecularly equivalent to the guanidine.

About 1 to 3 hours after injection, the animals receiving guanidine usually showed great excitement, continuous face-washing and rapid breathing. This face-washing movement is frequently seen in normal rats, but is there extremely spasmodic and does not continue over a prolonged period as in the rats intoxicated with guanidine. Salvesen *et al.* [1924] report similar symptoms observed by them in parathyroidectomised dogs and in dogs in which they had induced tetany by the injection of large quantities of phosphates. When the dose of guanidine is non-lethal, the symptoms of irritation and the salivation gradually cease, and the breathing becomes normal. Group B (Table IV) shows the muscle-calcium values for rats killed in the condition of hyperexcitability.

A condition of hyperexcitability similar to that of rats 2 hours after a non-lethal dose of guanidine was produced in rachitic animals made rachitic on a diet with high Ca/P ratio, by adding to the diet phosphate to give Ca/P = 1, or irradiated ergosterol. After about 2 days, the healing of the bone lesions was found to be accompanied by a definite increase in the excitability of the animals, more rapid breathing and much face-washing. After a few hours these symptoms disappeared.

In another group, animals on stock diets were given a lethal dose of guanidine, and were killed usually when the intense face-washing, salivation and gasping indicated that the animals were on the verge of acute generalised tetany. (300 mg. of guanidine per kg. of body weight was usually enough to produce these symptoms in 1–2 hours if given I.P.) Group C (Table IV) gives the results of these experiments in which acute symptoms were produced.

Table IV.

Ca in mg. per 100 g. fresh muscle.

Substance injected ...	None	Saline	Ammonium lactate	Sodium lactate	Guanidine lactate A	Guanidine lactate B	Guanidine lactate C
No. of rats...	10	5	5	15	7	9	9
Symptoms ...	None	None	None	None	Latent tetany	Hyper-excitability	Tetany
Max.	8·5	—	—	7·2	7·8	7·7	8·8
Min.	5·6	—	—	5·4	5·9	5·6	6·5
Av.	**6·4**	**5·2***	**5·9***	**6·4**	**6·4**	**6·7**	**7·2**
σ	0·8	—	—	0·54	0·66	0·70	1·07

* One estimation on pooled muscle from 5 rats.

Table IV does not indicate any difference in the total muscle-calcium of rats according to the degree of hyperexcitability of the rat.

In addition to inducing excitement in rachitic rats by dietary changes, tetany was produced by injecting guanidine into rachitic rats which had received additional phosphate for 2 days (healing rickets) and into a small batch which had received irradiated ergosterol for 4 weeks (healed rickets). In both these groups active tetany was produced in 30–60 mins. by doses of 200 mg. guanidine per kg. body weight, given I.P. In one or two animals half this dose sufficed to produce acute symptoms. It is not yet known whether this lack of resistance was a peculiarity of the group or was due to the diet.

Table V shows the results of the investigations on animals on rachitic diets.

Table V.

Ca in mg. per 100 g. fresh muscle.

	Rickets	Healed rickets	Healing rickets	Healing rickets + guanidine	Healed rickets + guanidine
No. of animals...	4	3	4	5	6
Symptoms ...	None	None	Excitement and twitching	Tetany	Acute tetany
Max.	5·6	7·4	5·8	7·8	7·0
Min.	5·1	5·8	4·2	4·6	5·0
Av.	5·4	6·8	5·0	5·6	5·8

These figures do not differ significantly from each other or from those for the muscle of normal rats of this breed.

The influence of the injection of calcium chloride was demonstrated in one experiment in which 7 rats suddenly developed acute tetany after a dose of guanidine which they had been tolerating for some time. Their death was not desired at the time, and in an attempt to bring about their recovery 2 cc. of 10 % $CaCl_2$ were injected into each animal I.P. The controls were similarly treated. A temporary improvement followed, but next morning five of the animals were found dead, and the other two were killed. The macroscopic appearance of the muscles of these animals was normal. Two days later littermates receiving saline were killed, and the following day controls to which ammonium lactate had been given were killed. In all the animals which survived more than 48 hours after the injection sores appeared round the site of injection, extending towards the hind-legs; the muscle still, however, appeared normal. The average values for the calcium contents of the muscles (mg. per 100 g. fresh tissue) for the respective groups were guanidine 23·3, saline 13·9 and ammonium lactate 9·7. At first it seemed that these differences were due to the influence of the guanidine injections, but careful study of the individual figures showed that all the higher figures except one (and that in the saline group) were from animals killed within 48 hours of the calcium injection. The development of the sores indicated that some of the calcium solution had leaked into the tissues, and it seemed probable that some of this calcium had diffused into the muscle, from which its removal was slow, so that the animals first killed showed on the whole the higher content of muscle-calcium. It may be calculated that the presence in 5 g. of muscle tissue of 0·07 cc. of the original injected calcium solution would suffice to raise the apparent calcium content of the muscle from 5 to 50 mg. per 100 g. fresh tissue, without raising the water content beyond normal limits. The water content of these muscles did not in fact vary with the calcium. An experiment was, therefore, carried out in which 5 rats which had been receiving small doses of guanidine were given 500 mg. of guanidine per kg. in 3 cc. of fluid I.P., and 1 hour later received 3 cc. of 5 %

$CaCl_2$ solution, also I.P. A batch of controls was similarly treated except that sodium lactate was substituted for the guanidine. All these latter animals remained normal, but all those receiving guanidine developed symptoms of acute guanidine intoxication. The animals were killed 1 hour after the injection of the calcium chloride. The muscle-calcium values were as follows, for the sodium group 17·2, 18·0, 29·1, 29·1, 26·3, and for the litter-mates of the guanidine group 20·6, 30·7, 63·3, 10·2 and 9·6 respectively. None of these animals had a high value for liver-calcium, which would have indicated that the calcium had been carried there by the blood, and it seemed most probable that these high muscle-calcium figures could best be explained by leakage of the injected calcium into the surrounding tissues.

A small group of 8 rats was used to test the effect of parathormone injections on muscle-calcium. Six control rats had muscle-calcium values ranging from 5·9 to 9·4 mg. per 100 g. fresh tissue with an average of 6·9, while 8 rats which received a total of 200 units of parathormone each in increasing doses over a period of 98 days had values ranging from 6·9 to 8·2, with an average of 7·2.

In none of these experiments on rats was muscle-calcium found to vary consistently from normal figures, except where calcium chloride solution had been injected into the peritoneum of animals which had already received intraperitoneal injections of considerable quantities of fluid. There is, moreover, no indication of any consistent difference from litter to litter. Nearly 200 rats were used altogether, but if from these are excluded all those which received calcium injections, and those in which the calcium estimations were done on the mixed muscle of several rats from different litters, there are left 138 estimations for the thigh muscles of rats from 23 litters. Of these 138, 3 are markedly different from the rest, *viz.* 13·6, 18·2 and 23·2 mg., and appear to have been influenced by some special factor. If they are arbitrarily excluded, and the average taken for the muscle-calcium of each litter, irrespective of the treatment which the animals have received, the following figures are derived.

Table VI.

Litter	Average	Litter	Average	Litter	Average	Litter	Average	Litter	Average
1	7·0	6	6·5	11	6·8	16	7·3	21	8·1
2	6·7	7	6·3	12	6·2	17	6·7	22	7·6
3	7·0	8	6·3	13	6·9	18	6·6	23	7·1
4	6·7	9	6·4	14	6·8	19	5·6		
5	6·2	10	6·7	15	6·2	20	5·9		

In Fig. 1 the distribution of the 138 estimations made on animals which received no injected calcium salts is indicated. The figure as a whole indicates that the greatest number of rats had a thigh muscle-calcium of from 6 to 6·5 mg. per 100 g. fresh tissue, and that a large proportion of the total lay between 5–8 mg. with a few more and a few less than this range. By the shaded portions of the graph is indicated how this total was made up from the different groups. All rats were classed as normal which received a complete diet, grew well, had normal bones and blood and showed no pathological symptoms, whatever they were receiving as injection (*i.e.* saline, ammonium lactate, *etc.*). The figure shows that the muscle-calcium of these 55 animals was almost equally distributed between 5 and 7·5 mg., but that there were a few figures outside this range. The group classed as "prevented rickets" which included animals on diets R + E, P + E and P are not included among normal animals as their growth was variable and on the whole poor, although their serum-calcium was normal, and bone calcification almost so. These 33 animals had a muscle-calcium range

from 6–8 mg. with few cases greater and none less. (The three very high figures occur in this group.) The four small groups, tetany (20 rats), latent tetany (9 rats), excitement (13 rats) and rickets (13 rats) seem to be almost equally distributed over the whole range. This may be due to greater variability among animals under pathological conditions, or may merely result from the small number of animals in each group.

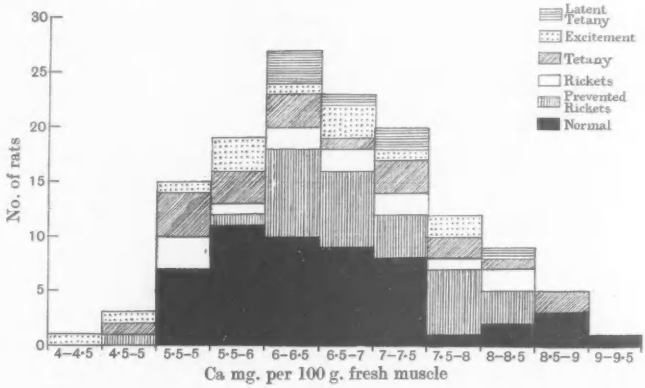

Fig. 1.

Dennis and Corley [1925] noted a similar variation in rabbit muscle and suggested as a probable cause the contamination of the muscle with lymph, blood, *etc.* Blood usually has a calcium content of 5–7 mg. per 100 cc. Two samples of fat from rats were examined for calcium and proved to contain 1–2 mg. per 100 g. (The amount of fat available was too small to give the calcium more accurately.) Three samples of sheep tendon were analysed and the calcium found to be 8·1, 7·5 and 9·4 mg. per 100 g. fresh tissues. (It was assumed that the connective tissue of rats and sheep would probably contain the same order of calcium.) Nerve tissue (brain) of rats was found in agreement with Dennis and Corley (for rabbits) to contain calcium ranging from 8 to 16 mg. per 100 g.

Now it may be calculated that if a muscle of actual calcium content of 8 mg. per 100 g. was estimated to have only 5 mg./100 g. of calcium because of the presence of fat containing 2 mg. per 100 g. of calcium, the latter would need to be present to the extent of 50 %. Similarly if a muscle with actual calcium content of 5 mg. appeared to contain 8 mg./100 g. because of contamination with nerve or connective tissue of a calcium content of 10 mg., then at least 60 % of nerve or connective tissue would have to be present. Actually the animals were all bled from the neck and drained thoroughly, and all visible fat, blood-vessels and nerve tissue were removed. The tendons were cut off, but no attempt was made to remove the thin fibrous and closely adherent sheath of connective tissue. It would, however, seem improbable that the major variations in muscle-calcium were due to contamination with other soft tissues. The bone-calcium of the animals examined varied from 4 to 15 % and contamination with a few mg. of such bone would be enough to raise muscle-calcium to very high figures. To prevent such contamination, the muscles were removed with great care, and the layer of muscle next the bone was never included in

the muscle for analysis. After dissection, all the muscle was carefully inspected through a hand lens. It would, therefore, seem more probable that the variations found in muscle-calcium represent real muscle differences rather than degrees of contamination.

Experiments on animals other than rats.

The difference between the calcium of the same muscle from opposite sides was studied in dogs, frogs and a cat. Muscles were taken in pairs from the two sides of dogs which had been used for blood-pressure measurements after small doses of guanidine, and the calcium was determined on each sample. A normal cat and three normal frogs were used in the same way (Table VII). Differences in the calcium contents of muscle from the two sides were here of the same order as had been previously noted for rats. The three samples of frog muscle examined were definitely richer in calcium than the muscle of the other species.

Table VII.

Ca in mg. per 100 g. fresh muscle.

	Dogs						Frogs			Cat
Right	4·1	5·3	4·4	4·2	5·0	4·3	12·4	23·7	18·0	5·6
Left	4·4	6·9	6·6	3·7	5·1	5·4	11·6	23·4	20·0	4·3

The effect of guanidine intoxication on rabbits was studied in two experiments. In the first, 6 rabbits were anaesthetised with ether, and the thigh muscles of one leg removed. 200 mg. of guanidine per kg. of body weight were injected intravenously as lactate (adjusted to p_H 7·0), and at the end of 20 mins., when an unanaesthetised animal would have been in acute tetany, the similar muscle from the other leg was removed. Muscle-calcium before and after guanidine was determined (Table VIII).

Table VIII.

Ca in mg. per 100 g.

Rabbit ...	I	II	III	IV	V	VI
Before	8·3	7·0	4·7	4·2	5·2	5·8
After	5·0	6·0	3·6	4·3	5·0	5·0

In the second experiment two litters of two and six respectively were used. One of the two rabbits was kept as control, and the other was given a dose of 300 mg. guanidine per kg. of body weight. The guanidine solution was prepared by bringing a solution of guanidine carbonate to p_H 7·0 with phosphoric acid. Three of the six were similarly injected with guanidine, one was kept as an uninjected control, and two received injections of sodium solutions prepared like the guanidine solution, so that the sodium was molecularly equivalent to the guanidine. At the end of 25 minutes all the four animals receiving guanidine were showing marked symptoms of tetany. At the end of 30 minutes, when all were in acute tetany and two were in opisthotonus, all the animals were killed. The two which received sodium were excited for a couple of minutes after the injection, then rapidly became normal. From 5 to 30 mins. after the injection they appeared perfectly well and normal and chewed fragments of hay that were lying about the cage. The calcium and solids of the serum and the calcium of the thigh muscles were determined for all these animals (Table IX). No consistent change was found in the muscle-calcium despite the violent muscular

contractions. Serum-calcium is only definitely lowered as compared with control animals when total serum-solids are also appreciably lowered, and this may, therefore, only indicate dilution of the blood by the injected fluid. In rabbits, as in rats, there is nothing to correlate the increased muscular excitability typical of tetany with any change in the total calcium content of the muscle.

Table IX.

	Control litter *a*	Guanidine *a*	Control *b*	Sodium *b* 1	Sodium *b* 2	Guanidine *b* 1	Guanidine *b* 2	Guanidine *b* 3
Serum-Ca (mg. per 100 cc.)	14·4	9·2	13·7	12·1	10·0	12·5	10·4	8·0
Serum-solids (g. per 100 cc.)	8·6	6·7	8·25	8·0	8·3	8·2	7·6	6·6
Muscle-Ca (mg. per 100 g.)	4·1	3·2	3·4	3·5	3·2	3·5	3·4	4·0

Parathyroidectomised kittens. The effect of parathyroidectomy on muscle-calcium was studied in four litters of kittens. Total thyroparathyroidectomy was performed under ether; in each experiment, one kitten from the litter was used as a control and was anaesthetised, a skin incision made and the thyroid sheath split and manipulated as though the thyroid was to be removed. The animals were fed on milk after the operation. Four of the kittens so thyroparathyroidectomised showed marked tremors after 40 hours and developed a quite characteristic paw-shake, as though water was being shaken off the paws. One kitten, at this stage, suddenly developed acute tetany. All five were killed at this early stage. A sixth kitten survived for a week without showing any symptoms of tetany and seemed quite happy throughout. It was killed on the seventh day, and *post mortem* examination showed that the operation for the removal of thyroids and the customary parathyroids had been successful. No examination of other tissues for accessory parathyroids was made.

In the fourth litter, two kittens developed acute tetany within 16 hours and were killed. The third parathyroidectomised kitten showed no symptoms of tetany during the three days succeeding the operation, but was then killed.

Table X.

	Control	Pd. 1	Pd. 2	Pd. 3	Control	Pd.
Serum-Ca (mg. per 100 cc.)	12·1	5·4	6·8	5·0	12·1	4·6
Muscle-Ca (mg. per 100 g.)	4·3	4·9	3·6	3·3	4·1	6·2
Symptoms	Nil	Tremors	Tremors	Tremors	Nil	Tremors

	Control	Pd. 1	Pd. 2	Control	Pd. 1	Pd. 2	Pd. 3
Serum-Ca (mg. per 100 cc.)	11·9	10·7	5·5	10·0	12·8	5·4	3·7
Muscle-Ca (mg. per 100 g.)	6·1	4·9	3·1	4·5	4·8	3·2	4·1
Symptoms	Nil	Nil	Acute tetany	Nil	Nil	Acute tetany	Acute tetany

Table X shows that while serum-calcium at the time of death varies with the state of tetany of the animal, no such consistent variation is found in muscle-calcium.

Discussion.

The experiments here described, on rats, cats and rabbits, broadly agree with the observations of Dennis and Corley [1925] that considerable variation may be met with in the normal muscle-calcium of animals, but that, at any

rate when control animals are receiving a normal amount of calcium in their food (in this case 0·4 %), further increase in the calcium or vitamin D of the diet does not increase muscle-calcium. The observations of Dixon, Davenport and Ranson [1929] on parathyroidectomised dogs are also confirmed on kittens. The results obtained, however, fail to confirm those of Haury [1930] on rachitic rats, or of Bülbring [1931] or of Loughridge [1926] on parathyroidectomised animals. It would seem that the latter might have reached a different conclusion after more experiments, but the results of Haury and of Bülbring cannot be so explained. The fact, however, that some animals seem to have markedly higher muscle-calcium values than the others of their species suggests that in these animals some special condition obtains. All Haury's control rats fell into this group, and it may be significant that the three very high figures found in the above investigation for thigh muscle of rats were for animals on diets somewhat similar to that chosen by him for his control group: *i.e.* diets producing the condition described above as "prevented rickets." No adequate comparison can be made between Bülbring's parathyroidectomised rats and the parathyroidectomised kittens of these experiments. Bülbring maintained her animals over the stages of tetany by injecting calcium solutions, while the kittens were killed in the early stages of the first attack of tetany. No conclusion can be drawn from these experiments as to the storage of calcium in the body after parathyroidectomy, but they seem to show that the muscular contractions of tetany in kittens are not preceded by any significant change in total muscle-calcium. This is also true of the tetany and muscular hyperirritability produced in rats and rabbits by guanidine intoxication or by the sudden induction of the healing processes in rachitic animals.

I wish to thank Mr A. L. Bacharach of the Glaxo Research Laboratory for the supply of irradiated ergosterol, and Prof. Burns and Dr Secker for parathyroidectomising the animals. The expenses of this research were defrayed by a grant to Prof. Burns from the Medical Research Council.

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V. NOTE ON THE PERMEABILITY OF ERYTHROCYTES TO CHLORIDE.

By MONTAGUE MAIZELS.

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(*Received November 21st, 1932.*)

When investigating the permeability of red cells suspended in mixtures of potassium phosphate and potassium chloride, it was found that the presence of chloride greatly depressed the permeation of the phosphate. At the same time the concentration of chloride in the red cells was in excess of that in the external fluid, and appeared to balance the total osmotic pressure of the external chloride and phosphate. In order to determine how far the external osmotic pressure was able to modify the anion content of erythrocytes, these cells were suspended in mixtures of potassium chloride with glucose, to the latter of which they are relatively impermeable. At the same time, observations of the change in p_H of the external fluid were made, to see how this was affected by the redistribution of Cl^-.

Method.

A modification of the haematocrit was used in these determinations. Blood containing heparin as an anti-coagulant was centrifuged in tubes drawn out into sealed, graduated and calibrated capillaries for 30 min. at 4000 r.p.m., after which the cells had attained constant volume (*ca.* 0·05 cc.) and were translucent. The volume of red cells was read, the supernatant plasma washed away, and the cells resuspended in 7 cc. of the test fluid. After 10 min. the tubes were respun and the volume of cells again read. The approximate p_H of the test fluid was determined colorimetrically, and then the tube and capillary down to the upper face of the red cell column were carefully washed with a capillary pipette. Lastly, the red cells were removed and their chloride content estimated by the method of Claudius [1924].

Among the constituents of the red cell are H^+, Cl^- and HCO_3^- which very readily permeate the cell membrane, and potassium and haemoglobin which do not. When such cells are placed in solutions of potassium chloride, the H^+, Cl^- and HCO_3^- are redistributed according to the Donnan equation,

$$\frac{[H^+]_{cell}}{[H^+]_{fluid}} = \frac{[Cl^-]_{fluid}}{[Cl^-]_{cell}} = \frac{[HCO_3^-]_{fluid}}{[HCO_3^-]_{cell}}.$$

As the volume of the external fluid is relatively very great Cl^- will largely displace HCO_3^-, and the HCO_3^- will combine with an equivalent amount of potassium in the external phase, where its concentration will necessarily be very low. When the Cl^- content of the external fluid is raised, water flows out of the cell and cell Cl^- rises. If no Cl^- enters the red cell from the external fluid beyond that required to displace HCO_3^-, and if allowances are made for changes in cell volume, then the Cl^- calculated as a content of the original volume of cells should equal at all dilutions the sum of HCO_3^- and Cl^-, which existed in the original untreated cell. Cell $[Cl^-]$ varies between 48 and 54 *mM*, and according to Henderson [1928], the cell $[HCO_3^-]$ is about 13 *mM*, corresponding to a total concentration of 61 to 67 *mM*. Table I shows that if

allowances are made for changes in volume, the chloride content of red cells suspended in potassium chloride solutions at p_H 7·6, is fairly constant within wide limits of dilution, and is approximately equal to the sum of the normal Cl^- and HCO_3^-.

Table I.

Cells in KCl solution at p_H 7·6.

External fluid $[Cl^-]$ *mM*	Cell $[Cl^-]$ *mM* (A)	Final volume. Original vol. = 100 % (B)	$\frac{A \times B}{100}$
89	46	140	64
124	58	113	65
145	63	102	64
216	85	82	70

If, as a result of dilution, the external $[Cl^-]$ fell very low, according to the Donnan equation Cl^- should leave the cell, and as Cl^- cannot diffuse alone, H^+ would have to leave with it; that is, a hydrolysis of KCl inside the cell should occur resulting in a decrease of cell $[Cl^-]$ and external p_H, and the combination of the corresponding cell K with haemoglobin. Owing to haemolysis, the direct observation cannot be made, but Coulter [1924–5] has shown that if cells are placed in a solution of sucrose, the latter becomes more acid. This experiment was confirmed by placing 0·1 cc. of cells in two tubes and adding 10 % glucose at p_H 7 to one, and 1·7 % KCl at p_H 7, to the other; the p_H of the glucose changed to 5·8, and that of the KCl to 7·6. At the same time the cells in glucose lost 10 *mM* of chloride, which corresponds to an external p_{Cl} of 4·2. There is thus some discrepancy between the external p_H and p_{Cl}.

Red cells were next placed in a solution of 24 *mM* KCl in 9 % glucose at p_H 7. The p_H of the external solution rose to 7·6 and the $[Cl^-]$ of the cells to 79 *mM*, the internal chloride was therefore much higher than the external chloride, and was nearly as great as the $[Cl^-]$ of cells exposed to a pure KCl solution of the same osmotic pressure as that of the glucose and KCl in the experimental solution.

When cells are placed in glucose solution containing HCl, a proportion of the H^+ and Cl^- enters the cells. If the HCl is kept constant and KCl is added to the outer phase, cell $[Cl^-]$ must increase correspondingly, in accordance with the Donnan equation. As the potassium of the KCl cannot permeate readily, cell Cl^- does not diffuse from the external KCl, but is mainly derived from, and limited by, the external HCl. It follows that with increasing concentration of external KCl, more and more HCl will pass into the red cell, and the external p_H will rise. This effect is shown in Table II.

Table II.

	External fluid				Cells	
Exp. No.	Cl as HCl *mM*	Cl as KCl *mM*	Glucose %	Final p_H	Cl *mM*	Volume (normal = 100 %)
1	1	0	10	3·6	86	78*
2	1	1	10	3·8	100	78*
3	1	4	10	4·1	124	83*
4	1	24	9	4·7	168	85*
5	1	115	5	5·0	174	99
6	1	230	10	5·0	240	71
7	1	230	0	5·2	176	104

* These volumes are approximate owing to cell agglutination and partial distribution on the wider part of the centrifuge-tube.

It will be seen that the external p_H increases with the addition of KCl, and that the [Cl^-] of the cells is far in excess of the external [Cl^-]. If glucose diffused freely, then cell [Cl^-] would depend only on the external [Cl^-] and p_H, and the anomalous distribution of Cl^- actually found must be correlated with the observation that glucose permeates the red cell very slowly. Under these circumstances it appears that the [Cl^-] in the cell depends on the p_H and the osmotic pressure of the external non-permeating substance, provided that a large excess of total chloride is available, and a certain relatively low minimum concentration is exceeded (Exps. 4, 5, 6 and 7). In interpreting cell Cl^- changes, it must be remembered that untreated red cells have a [Cl^-] of 50 *mM* per litre and also that their final volume is less than 1/140 of the external fluid. Thus in Exp. 1 the gain in cell Cl^- is about 12 % of the external Cl^- and in Exp. 4, about 70 % of the external HCl and 3 % of the total Cl^-. The loss of external Cl^- in Exp. 1 is equivalent to a rise in p_H from 3 to 3·1 (approximately). The discrepancy between this and the actual p_H recorded (3·6), may be due in part to the buffering action of that fraction of HCO_3^- displaced from the cell by chloride and also to the red cell not being completely impermeable to cations.

In this type of experiment, the external osmotic pressure may be maintained by a non-electrolyte, like glucose, or by a slowly penetrating anion, such as phosphate. In mixtures of potassium phosphate and chloride, the presence of the Cl^- greatly depresses the permeation of phosphate, and excess of Cl^- enters the red cell to balance the external phosphate and chloride. This effect is best seen at p_H 5·1, where the cell is least permeable to phosphate, and is less evident at p_H 6, where the cell is more permeable. Cells were exposed for 5 min. and the temperature was kept constant, because it was found that phosphate diffusion has a high temperature coefficient (2·4 between 20 and 30°).

Table III. *Diffusion of chloride from phosphate-chloride mixtures of varying strengths.*

Depression of freezing-point, 0·42°.

Cl/phosphate by volume	External Cl^- *mM*	Internal Cl^- *mM*	External phosphorus mg./100 cc.	Internal P mg./100 cc.
1/0	124	112	0	0
1/1	62	110	200	22
1/6	21	102	330	39
0/1	0	40	400	170

The phosphate-chloride equilibrium is unstable, because with the lapse of time more phosphate enters the red cell and Cl^- is displaced.

Conclusion.

If red cells are exposed to solutions containing permeating anions and other anions or non-electrolytes which permeate less easily, the amount of permeating ion diffusing across the positively charged membrane into the red cell depends on the external p_H and the total osmotic pressure of the less diffusible substances—always provided that a certain absolute quantity and minimum concentration of the permeating ion is exceeded. The concentration of this ion in the cell may be many times greater than its equilibrium concentration in the external phase.

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VI. A STUDY OF THE OXIDATION OF 3:4-DIHYDROXYPHENYL-*N*-METHYLALANINE WITH REFERENCE TO ITS POSSIBLE FUNCTION AS A PRECURSOR OF ADRENALINE.

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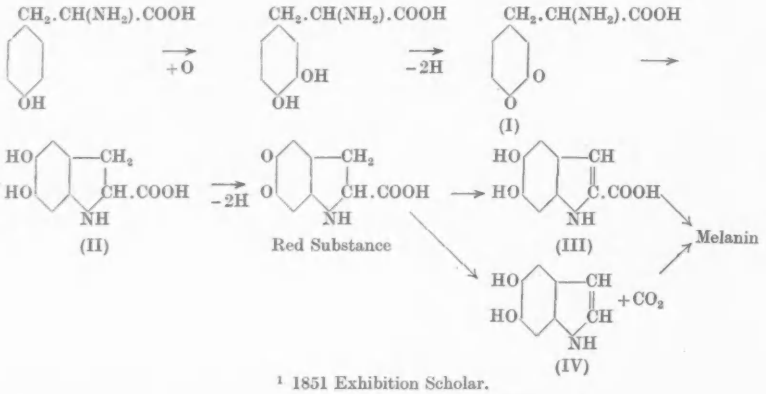
(*Received November 26th, 1932.*)

The close structural relationship which adrenaline bears to two amino-acids which are found in proteins suggests that either of them may logically be regarded as its parent substance. These two amino-acids, phenylalanine and tyrosine, possess almost the same carbon skeleton as adrenaline and might give rise to the latter by successive oxidation, methylation of the nitrogen atom and loss of CO_2. Investigators have from time to time unsuccessfully attempted to demonstrate a synthesis of adrenaline in this manner. Hallé [1906] exposed the adrenal glands freshly removed from the body to solutions of tyrosine and by gravimetric assay claimed to have obtained an augmentation in adrenaline content. Ewins and Laidlaw [1910] repeated and extended the experiments of Hallé, applying specific biological methods to the estimation of adrenaline. It was conclusively shown that no stage requisite to the synthesis of adrenaline from tyrosine is brought about under these conditions. The failure to obtain direct evidence of such by the action of surviving adrenal tissue does not mean that the intact organ cannot accomplish this synthesis or that the hypothesis as to the gross chemical mechanism is wrong; neither is there any evidence to suggest that all or even part of these requisite changes need take place within the adrenal gland.

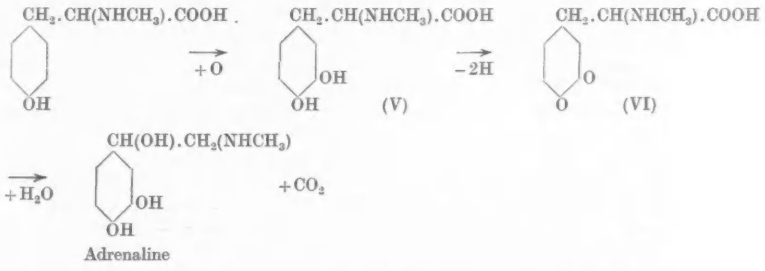


[1] 1851 Exhibition Scholar.

The isolation of *l*-3 : 4-dihydroxyphenylalanine (dopa) from the pods of *Vicia faba* by Guggenheim [1913] revealed another possible precursor of adrenaline. Renewed biological interest attached itself to this amino-acid, and the first obstacle in accepting the view that tyrosine is the parent substance of adrenaline was overcome when it was shown that dopa represents the first stage in the formation of melanin from tyrosine by the action of the enzyme tyrosinase [Raper, 1926].

The scheme of reactions given on p. 36 represents the changes which take place when tyrosine is oxidised to melanin [Raper, 1927].

The oxidation of a methylated tyrosine, which is still more closely allied to adrenaline, was found to proceed along slightly different lines from the above [Dulière and Raper, 1930]. Judging by the yields of indole derivatives corresponding to (III) and (IV) obtained from *N*-methyltyrosine on oxidation with tyrosinase, the quinone corresponding to (I) does not change immediately into the corresponding 5 : 6-dihydroxydihydroindole-2-carboxylic acid derivative (II) as with tyrosine. On allowing the red solution from *N*-methyltyrosine to decolorise *in vacuo* it was found that a small amount of pressor substance is produced. The following scheme of reactions was therefore postulated by which the quinone corresponding to (I) might conceivably give rise to adrenaline by an internal oxidation-reduction process and loss of CO_2.



The production of the 3 : 4-quinone of phenyl-*N*-methylalanine (VI) is in full accord with what is already known of the action of tyrosinase, and, with the object of investigating in more detail the changes which this quinone undergoes, the intermediate compound (V), 3 : 4-dihydroxyphenyl-*N*-methylalanine (*N*-methyldopa), has been submitted to the action of the enzyme.

The initial point of attack in the *N*-methyltyrosine molecule, when it is oxidised by tyrosinase, is the *ortho*-position to the phenolic hydroxyl group. This oxidation requires several hours. With the *ortho*-dihydric substrate oxidation takes place within a few minutes and gives rise to a greater amount of the red substance. Consequently the pressor activity of the solution from *N*-methyldopa when allowed to decolorise *in vacuo*, is considerably in excess of that obtained with *N*-methyltyrosine. Attempts to ascertain those conditions which would limit indole formation and promote greater side-chain oxidation with the production of an increased yield of pressor base met with little success. In each case it was found that the rise in blood pressure obtained on injection of the decolorised solution is not typically adrenaline-like in nature and may be imitated by administration of small doses of the ketone adrenalone. On reduction of the decolorised solution by catalytic hydrogenation a large increase in pressor activity develops. The reduced substance behaves in all respects like

adrenaline and shows the customary adrenaline reversal subsequent to administration of ergotoxine. Since no known pressor base other than a ketone of the adrenalone type exhibits this phenomenon, it is almost certain that the active substance obtained from *N*-methyldopa is adrenalone. It was found that this ketone, unlike adrenaline, is not oxidised by tyrosinase and therefore may accumulate in the reaction solution as the end-product of a secondary oxidation which occurs simultaneously with those primary oxidative changes which are concerned with the conversion of the substrate into a methylated melanin.

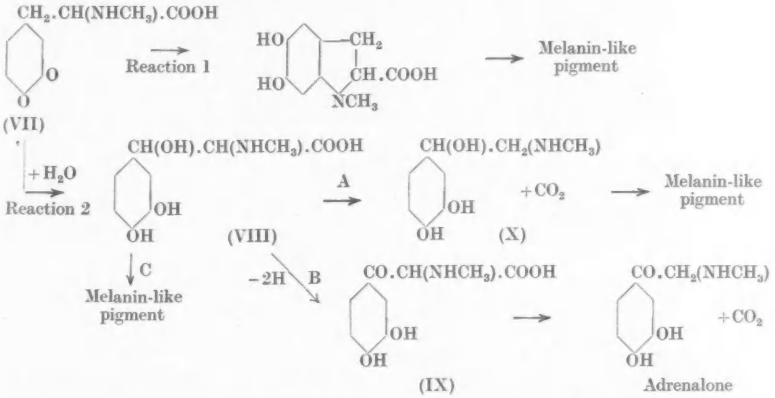
The oxidation of dopa was investigated in a similar manner, when it was found that this substance, like the methylated amino-acid, also gives rise to a pressor base which exhibits increased activity on reduction. The yield of active substance, presumably aminoacetocatechol, and the increase in activity obtained on its reduction to *nor*adrenaline are less than with *N*-methyldopa. A respirometric comparison of the rates of oxidation of these two substrates shows that both the production of the red substance and its subsequent oxidation to a melanin take place more slowly with *N*-methyldopa. Methylation of the amino-nitrogen atom diminishes the velocity of the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine (I) is converted into the corresponding dihydroindole derivative (II) and thereby increases the time interval during which the quinone (I) may undergo side-chain oxidation.

It appeared conceivable that complete methylation of the amino-nitrogen atom, which prevents indole formation, would tend to promote quantitative side-chain oxidation. In the absence of a dimethylamino-acid of this type, the oxidation of hordenine with tyrosinase was investigated. This *N*-dimethyl base does not give rise to the corresponding ketone. Neither can the production of adrenalone be demonstrated on oxidation of adrenaline and 3:4-dihydroxyphenylethylmethylamine (epinine) with tyrosinase. Obviously β-oxidation in the side-chain takes place only when the terminal carboxyl group is retained; a fact which emphasises the importance of the corresponding β-hydroxy- and β-ketonic acids and may possibly explain the physiological inactivity of the precursors of adrenaline in the body.

Of the series of reactions which takes place when tyrosine is acted upon by tyrosinase, the only specific one is the introduction of a second hydroxyl group in the *ortho*-position [Dulière and Raper, 1930]. The subsequent oxidation of this product may be brought about by any enzyme or agent that gives rise to *ortho*quinones from catechol derivatives. Consequently the oxidation of dopa and its *N*-methyl derivative with silver oxide, iron and hydrogen peroxide and peroxidase and hydrogen peroxide was investigated. With the former two agents *N*-methyldopa gives rise to a solution which exhibits a typical adrenaline-like rise in blood pressure which is not increased in activity on reduction. Adrenalone, like adrenaline, is rapidly oxidised and inactivated by these agents and may not accumulate as in oxidations with tyrosinase. The pressor activity obtained from *N*-methyldopa on oxidation with silver oxide and with iron and hydrogen peroxide is almost certainly due to the presence of a small trace of adrenaline. Dopa behaves in an analogous manner, but yields a smaller amount of active substance than with tyrosinase. With peroxidase and hydrogen peroxide, which oxidise adrenalone more slowly, the evidence obtained suggests that *N*-methyldopa produces small amounts of both adrenalone and adrenaline. It was found that in this case the oxidation of dopa proceeds along different lines and gives rise to little or no pressor base.

The series of reactions which interprets these results most satisfactorily may

be represented by the following scheme which shows the changes that the 3:4-quinone of phenyl-*N*-methylalanine most likely undergoes.



The total oxygen uptake of *N*-methyldopa on oxidation with tyrosinase indicates that the greater part of the quinone (VII) is converted into a dihydroindole derivative which is subsequently oxidised to a methylated melanin (Reaction 1). A portion however gives rise to adrenalinic acid (VIII) by an internal oxidation-reduction process involving the addition of the elements of water (Reaction 2). The β-hydroxy-acid (VIII) is partially converted into a melanin-like pigment (Reaction 2 C) by a series of changes analogous to Reaction 1, whilst the remainder is oxidised to the corresponding β-ketonic acid (IX) by removal of 2 hydrogen atoms (Reaction 2 B). Adrenalonic acid (IX) gives rise to the stable ketone adrenalone on loss of CO_2. With tyrosinase the yield of adrenalone, which has been found to represent at least 3 to 7 % of the original substrate, is not necessarily a measure of the amount of the quinone (VII) which enters into Reaction 2; this may be much greater depending upon the relative velocities of Reactions 2 B and 2 C. The ketone is readily oxidised by silver oxide and by iron and hydrogen peroxide with the ultimate production of a melanin-like pigment and may not accumulate as with tyrosinase. In this case the presence of a small amount of adrenaline (X) is demonstrable (Reaction 2 A). The results suggest therefore that with tyrosinase the reactions taking place subsequent to the formation of the 3:4-quinone of phenyl-*N*-methylalanine (VII) are 1, 2 B and 2 C, the products being a melanin and adrenalone. With silver oxide or iron and hydrogen peroxide the reactions concerned are 1 and 2 A and 2 C, and the products are a melanin and adrenaline. With peroxidase and hydrogen peroxide it would appear that the reactions concerned are 2 A, 2 B, and 2 C, the products being a melanin, adrenaline and adrenalone. The active bases, adrenaline and adrenalone, are produced in small amounts because the former is oxidised by all oxidising systems employed and the latter by all except tyrosinase and atmospheric oxygen.

On perfusion of the adrenal gland with *N*-methyldopa, it has not been possible to demonstrate any production of adrenaline. The substrate is however extraordinarily stabilised on perfusion and thereafter resists oxidation by tyrosinase for a long period of time. With the unmethylated amino-acid the same phenomenon takes place. The organ has been perfused before by Schkawera

and Kusnetzow (1923), who described the presence of a remarkably stable "adrenaline-like substance" in the Ringer-Locke perfusate. They concluded that a stable precursor of the hormone was washed from the gland. In view of the resistance to oxidation of the catechol amino-acids after perfusion, this appears unlikely. Adrenaline, when added to the Ringer-Locke perfusate, was found to retain its pressor activity on remaining in the alkaline medium for many days. It is more probable therefore that the "adrenaline-like substance" of Schkawera and Kusnetzow is in reality a stabilised adrenaline. Conceivably oxidation may be delayed either by union of the catechol substrate with a suitable compound or by a continuous and complex interplay of chemical reactions constituting an oxidation-reduction system. The recent investigations of Wiltshire [1931] have shown that the presence of amino-acids delays the oxidation of adrenaline, and those of Szent-Györgyi [1928] have elucidated in part the mechanism of the oxidation-reduction system of the adrenal gland. The latter has isolated a hexuronic acid from the cortex and demonstrated the presence of a similar substance of high reduction potential in the medulla. The hexuronic acid molecule contains 2 labile hydrogen atoms which are readily released to a suitable hydrogen acceptor.

Szent-Györgyi has investigated the behaviour of coupled systems and shown that adrenaline is not oxidised as long as hexuronic acid remains present in the reduced form. It is assumed that the 3:4-quinone of adrenaline functions as a suitable hydrogen acceptor and is therefore reduced to adrenaline before the intramolecular change to the indole derivative may take place. He has shown that oxidised hexuronic acid may be reduced by the action of the glutathione system of a tissue complex and therefore, in a system of normal cell constituents, this stabilisation of catechol substance may be maintained indefinitely. Under aerobic conditions hexuronic acid is irreversibly oxidised by atmospheric oxygen and consequently the process is limited. This reducing system of the adrenal gland does not bring about the reduction of adrenalone on perfusion.

The apparent absence of tyrosinase from the higher animals must not be considered an obstacle to the acceptance of the general hypothesis of the synthesis of adrenaline from tyrosine or dopa. The only specific action of tyrosinase, namely the introduction of the *ortho*-hydroxyl group, may be brought about by other agents and is known to take place in the body. Recently the synthesis of dopa from tyrosine has been accomplished by the action of iron and hydrogen peroxide [Raper, 1932], and Medes [1932] has isolated *l*-dopa from the urine of a patient suffering from faulty tyrosine metabolism (tyrosinosis). That methylation of a nitrogen atom may be brought about in the animal body has been satisfactorily proven. The occurrence in nature of *l*-*N*-methyltyrosine as the active principle (surinamine) of *Geoffroya surinaminensis* [Winterstein, 1919] is of significance; possibly *N*-methylamino-acids may be normal protein constituents. The oxidation of the *ortho*-dihydroxy-substrate is occasioned by any agent which produces *ortho*quinones from catechol derivatives. A specific dopa oxidase is known to exist in the skin [Bloch, 1916], and the occurrence of peroxidases throughout the body is widespread. There are thus reasons for believing that the agents necessary for the production of adrenaline from tyrosine by one or other of the mechanisms which are described in this paper may be encountered in the body. As to whether the complete synthesis is brought about in the adrenal gland, there is no positive evidence.

The fact that the 3:4-quinone of phenyl-*N*-methylalanine changes so readily into a dihydroindole derivative and thence to a melanin and that the reactions which lead to the production of adrenalone or adrenaline take place only to a

small extent requires further comment. The change to the indole derivative may be prevented by complete substitution of the amino-nitrogen atom as would be found in an *N*-methyl peptide. This would give more opportunity for oxidation in the side-chain. There is no reason to believe that such substitution would inhibit oxidation by tyrosinase, since Abderhalden and Schairer [1931] have shown that tyrosine-containing peptides are oxidised by tyrosinase to a melanin-like pigment. The matter will, however, be further investigated.

Experimental.

In all experiments the estimation of pressor activity was made by intravenous injection into a decerebrate cat. The animals were prepared by, and the assays carried out in conjunction with, Dr W. Schlapp, to whom we wish to acknowledge our indebtedness.

Catalytic reduction of adrenalone and N-*dimethylaminoacetocatechol.*

Adrenalone was prepared by the method of Friedmann [1906]. A small amount of the *N*-dimethyl base was synthesised in an analogous manner. In all instances the ketones were employed as the hydrochlorides which contain 1 mol. of water of crystallisation.

Hydrogenations were carried out in a Thunberg tube of 15 cc. capacity which contained finely divided palladium black (approximately 0·05 g.) suspended in an aqueous solution of the ketone (5 cc.). The tube was evacuated, filled with purified electrolytic hydrogen to a positive pressure of 3 cm. of mercury, and mechanically shaken for 10 mins., after which the absorbed gas was replaced. This operation was repeated several times until the pressure of hydrogen inside the tube remained unchanged. The suspension was finally shaken for 30 mins., the catalyst removed and the solution assayed. The increase

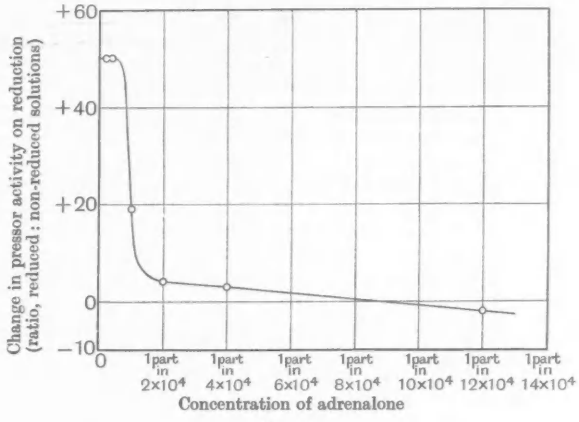


Fig. 1.

in pressor activity on reduction of adrenalone was found to be proportional to the concentration of the ketone employed. Consideration of the curve (Fig. 1) shows that in concentrations less than 1 in 4000 the amount of active substance adsorbed by the catalyst is no longer negligible and consequently the increase

in activity on hydrogenation falls off very rapidly with diminishing concentration. On shaking a solution of adrenalone (1 in 40,000) with palladium in the absence of hydrogen, it was found that the ketone was adsorbed to the extent of 50 %. The minimum concentration of adrenalone which can be detected by this method is obviously limited. The average ratio of the pressor activity of *l*-adrenaline to that of adrenalone was determined from a series of comparisons on different animals and found to be 100. Since *l*-adrenaline is approximately 1·5 times more active than the *dl*-isomeride, an increase in activity of 50 times on hydrogenation of adrenalone represents nearly quantitative reduction. The increase in activity on reduction of *N*-dimethylaminoacetocatechol (1 in 2000) was found to be 100 times. The ratio of the activity of the reduced product, *dl*-*N*-dimethyl*nor*adrenaline to that of *dl*-adrenaline is thus approximately 1:30.

Oxidations with tyrosinase.

The enzyme used in the following experiments was prepared from mealworms as decribed by Pugh [1930]; it was not dialysed before use unless so stated. Controls with the enzyme solution alone exhibited no pressor activity unless the tyrosinase had been prepared for more than a fortnight, when a histamine-like response was noted. Fresh preparations of the enzyme were therefore constantly employed and each experiment was rigidly controlled.

In most experiments the same general procedure was adopted, and unless otherwise stated the oxidation was carried out as follows. The substrate (10 mg.) was dissolved in hot water (5 cc.) and cooled, and phosphate buffer solution (4 cc.) was added. Oxidation with tyrosinase (1 cc.) was now allowed to proceed until a good red colour had developed and the enzyme had begun to precipitate. This procedure usually required from 15 to 30 mins. A few drops of 10 % acetic acid were then added to ensure complete precipitation of the enzyme, and the solution was allowed to stand for a few moments and then filtered. Decoloration of the red filtrate was carried out in one of two ways. When effected at room temperature the solution was placed in a Thunberg tube which was several times evacuated and filled with hydrogen and then set aside for 2 or 3 days. Alternatively decoloration was effected by heating in an inert atmosphere. The red solution was placed in a small flask through which hydrogen was passed until all air was expelled, when it was cautiously heated until decolorised. This phenomenon took place before the boiling-point was reached. The decolorised solution was then either tested for pressor activity immediately or divided into two equal portions and placed in Thunberg tubes. In the latter instance, one portion was catalytically hydrogenated, as described in the previous section, whilst the other was preserved under hydrogen until injections were made. When a quantity of substrate other than 10 mg. was oxidised, the amounts of water, buffer, and enzyme employed were proportional to the above values.

N-Methyldopa. *N*-Methyldopa was synthesised as already described [Heard, 1933]. Many preliminary experiments were carried out in an endeavour to ascertain those factors which influence the production of a pressor base. The reaction of the medium, which is known to have a marked influence on the velocity of the enzyme action and also on the nature of the pigments produced [Raper and Wormall, 1923], was investigated by oxidising *N*-methyldopa at p_H values 6·5, 7·0 and 7·7. The red filtrates were decolorised at room temperature for 3 days, when comparisons were made by intravenous injection. The solution oxidised at p_H 6·5, which is optimum for the production of the

red quinone, exhibited a slightly greater pressor response. The influence of reaction on the decoloration of the red substance was investigated in the following manner. *N*-Methyldopa (25 mg.) in water (22 cc.) was oxidised with tyrosinase (3 cc.), the p_H being adjusted to 6·5 by the cautious addition of a few drops of dilute ammonia (0·1 %). Aliquot samples (3 cc.) of the red filtrate were placed in six Thunberg tubes, each of which contained phosphate buffer (0·75 cc.). Decoloration was effected at room temperature over a p_H range of 4·5 to 9·5. The red substance decolorised in alkaline media overnight whereas in acid media the final disappearance of the red tint did not take place until the second day. On injection of the decolorised solutions no difference in pressor activity was noted. When decoloration was effected by heating, the yield of pressor base was increased by approximately 25 %. Consequently, in all succeeding experiments, oxidation was carried out at p_H 6·5 and the red substance was decolorised by heating in an inert atmosphere.

The above experiments were repeated several times and in each case the yield of pressor substance obtained was very small. The results were not always consistent and in a few cases injection of the decolorised solution did not cause a rise in blood pressure. Usually the pressor activity was found to be equivalent to that given by 1 in 30 millions adrenaline, but varied considerably from this value to zero. One comparison was made with the decolorised solution from *N*-methyltyrosine, which gave a pressor response equivalent to that shown by 1 in 50 millions adrenaline. On oxidation of the *ortho*-dihydric substrate a bright red solution was obtained in 20 mins. whilst with *N*-methyltyrosine only a fair red colour developed after 3 hours. In all cases the pressor responses obtained were not typically adrenaline-like in nature. The gradual rise in blood pressure, which followed a noticeable latent period, was more prolonged than that shown by adrenaline and could be imitated by administration of minute doses of the ketone adrenalone. Consequently the effect of catalytic hydrogenation on the pressor activity of the decolorised solution was investigated.

The red solution from *N*-methyldopa (15 mg.) was decolorised by heating in an atmosphere of hydrogen and divided into two equal portions. One was preserved under hydrogen until injections were made, whilst the other was reduced by catalytic hydrogenation. On removal of the catalyst previous to the injection of the reduced solution, it was noted that the latter rapidly became coloured red. Hydrogenation in this manner also effects the reduction of 5:6-dihydroxy-*N*-methylindole or its 2-carboxylic acid to the corresponding saturated dihydroindole derivative, which is rapidly oxidised by atmospheric oxygen to the red quinone. This may be adduced as confirmatory evidence supporting the structure of the indole derivative and the red substance. This experiment was repeated 8 times and in each case a large increase in pressor activity was obtained on reduction of the decolorised solution. The response became typically adrenaline-like in nature in that the rise in blood pressure was more rapid and less prolonged.

Injection of the reduced decolorised solution subsequent to the administration of ergotoxine caused a fall in blood pressure similar to that obtained with adrenaline under the same conditions. The pressor response was judged equivalent to that given by 1 in 500,000 adrenaline. Even when the original decolorised solution exhibited little or no pressor response, the presence of the ketone was strikingly demonstrated on hydrogenation when injection of the reduced solution caused a marked rise in blood pressure. Typical examples of the pressor responses obtained on injection of the decolorised solution before and after reduction are illustrated in Figs. 2 A and 2 B.

In order to measure more precisely the increase in activity on reduction, the hydrogenation of a concentrated decolorised solution was attempted. *N*-Methyldopa (150 mg.) in water (230 cc.) and phosphate buffer (52 cc.) at p_H 6·5 was oxidised with tyrosinase (18 cc.) in the usual manner. Oxygen was bubbled through the liquid which was frequently shaken until a good red solution was obtained (30 mins.). The bright red filtrate was concentrated by vacuum distillation in a stream of hydrogen until the fluid measured 15 cc.

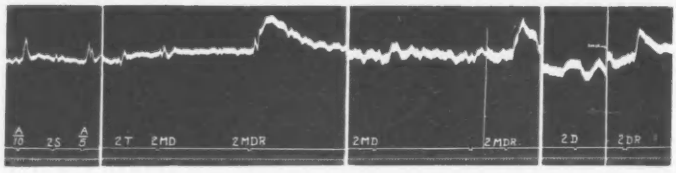


Fig. 2 A Fig. 2 B Fig. 2 C

Fig. 2. Oxidations with tyrosinase.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 D—2 cc. of decolorised solution from dopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.
2 DR—2 cc. of reduced decolorised solution from dopa.
2 T—Tyrosinase control.
2 S—2 cc. of physiological saline solution.

A and B illustrate the striking increase in activity developed on reduction of the decolorised solution from *N*-methyldopa. The pressor base originally present in the non-reduced solution is apparently the ketone adrenalone. C. With dopa the increase in activity on reduction is less marked. Presumably the non-reduced base is aminoacetocatechol.

approximately. The concentrate, which gave a strong indole reaction with *p*-dimethylaminobenzaldehyde, was divided into two portions. One was hydrogenated whilst the other was preserved under hydrogen until assayed. Both showed the same response on injection. It was not possible to estimate the amount of adrenalone present, as the pressor activity of the latter was masked by the presence in greater concentrations of toxic substances which caused a fall in blood pressure. The animal withstood the administration of this concentrate (1 cc.) only with difficulty. The reduced concentrate was not autoxidised with the formation of the usual red quinone, and therefore it appeared probable that hydrogenation had not taken place. A solution of adrenalone (2 cc. of 0·5 %) was added to the concentrate (2 cc.) and reduction was again attempted. The added ketone was not reduced. Obviously mealworm tyrosinase contains toxic substances which in relatively high concentrations cause a fall in blood pressure and poison the palladium catalyst. The experiment was repeated, using less enzyme (10 cc.), but the toxic substances still persisted.

It has not been possible as yet to estimate with accuracy the amount of adrenalone produced, but a minimum figure may be deduced. The minimum dose of adrenalone to which a definite pressor response is obtained was found to vary considerably with different animals. Usually in response to an injection of 1 cc. a concentration of 1 in 30,000 was required, although in a few isolated cases 1 in 75,000 sufficed. It has been quoted above that adrenaline is 100 times more active than adrenalone; this ratio was obtained by comparison of injections which gave a rise in blood pressure of approximately 30 mm. The ketone

is not a true sympathomimetic base, and this ratio does not hold good when adrenalone is administered in amounts approaching the minimum dose. Animals often respond to the injection of 1 in 100 millions adrenaline (1 cc.), whilst we have not observed a rise in blood pressure from adrenalone in doses less than 1 in 75,000 (1 cc.). The amount of adrenalone in the decolorised solution from *N*-methyldopa (0·1 %) closely parallels the minimum quantity to which a definite pressor response is obtained. This accounts for the inconsistent results obtained on injection of the non-reduced solutions and represents a conversion of from 3 to 7 % of the substrate. On the other hand it has been shown that hydrogenation of the ketone in a concentration of 1 in 75,000 results in only a twofold increase in pressor activity, and in a concentration of 1 in 30,000 an increase of approximately 6 times. While it has not been possible to estimate with accuracy the increase in activity on reduction of the experimental solutions, it may be seen from Fig. 2 that this value is considerably in excess of the above, which suggests that as much as 15 or 20 % of the substrate may be converted into adrenalone.

Dopa. In the manner described above the oxidation of the unmethylated substrate was also investigated. It was found that dopa is more rapidly oxidised and gives rise to a less stable red substance. Injection of the decolorised solution resulted in a rise in blood pressure almost as great as that obtained with *N*-methyldopa. On hydrogenation a striking increase in activity developed (Fig. 2 C). This was however less marked than that found with the methylated substrate. The pressor activity of the reduced solution was judged equivalent to that given by 1 in a million adrenaline. The pressor bases from dopa, presumably aminoacetocatechol and *nor*adrenaline, are more active than adrenalone and adrenaline respectively. Barger and Dale [1910] record the ratio of the activity of *dl*-adrenaline to aminoacetocatechol as 23:1 and to *dl-nor*adrenaline as 1·40:1. Aminoacetocatechol is therefore three times more active than adrenalone, and consequently the yield of the ketone from dopa is approximately one-third of that obtained from its *N*-methyl derivative. Methylation of the amino-nitrogen atom diminishes the velocity of the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine undergoes internal condensation in the 6-position with the production of a dihydroindole derivative, and therefore promotes oxidation in the side-chain.

Adrenalone. The hydrochloride (5 mg.) in phosphate buffer (10 cc.) at p_H 6·5 was treated with tyrosinase (1 cc.) for 30 mins., after which the enzyme was precipitated by the addition of a few drops of 10 % acetic acid and the light brown filtrate assayed. After treatment in this manner the ketone was found to have retained completely its original activity. The presence of the carbonyl group undoubtedly stabilises the hydrogen atoms of the hydroxyl groups and prevents quinone formation.

Hordenine. It appeared conceivable that a fully methylated base of this type, which is unable to undergo ring formation, might be oxidised almost quantitatively to the corresponding ketone, *N*-dimethylaminoacetocatechol. Hordenine sulphate (25 mg.) in phosphate buffer (25 cc.) at p_H 6·5 was oxidised with tyrosinase (3 cc.) for 12 hours. The solution darkened but no development of the usual red coloration took place. The enzyme was then precipitated by the addition of 10 % acetic acid and a portion of the brown filtrate, which gave an intense catechol reaction with ferric chloride, was catalytically hydrogenated. No increase in pressor activity developed on reduction. The rise in blood pressure caused by injection of the oxidised solution was approximately equivalent to that given by the original solution of hordenine sulphate.

Adrenaline. The free base (10 mg.) was dissolved in dilute hydrochloric acid (2 cc. of 1 %) and the solution neutralised to litmus by the addition of a few drops of dilute ammonia (1 %). The p_H was then adjusted to 6·5 with phosphate buffer (7 cc.), when the enzyme (1 cc.) was added. A bright red coloration developed immediately and after 30 mins. the enzyme was precipitated. The blood-red filtrate was allowed to remain under hydrogen for 3 days, when the red tint was replaced by reddish-brown and there was deposited a considerable amount of black melanin-like pigment. It was found that, regardless of extra precautions taken to remove as much oxygen as possible, this pigment formation could not be prevented. The oxidation of adrenaline does not proceed along the same lines as those of other substances already described, and it is conceivable that its 3:4-quinone is capable of bringing about anaerobic oxidation of the indole derivative to this melanin-like pigment. The solution which had stood for 3 days gave a strong indole test with *p*-dimethylaminobenzaldehyde, and on intravenous injection it was found that adrenaline when oxidised in this manner is 97 % inactivated. The residual pressor activity was not enhanced on hydrogenation of the solution, thus indicating that adrenalone was absent.

3:4-Dihydroxyphenylethylmethylamine (*epinine*). The hydrochloride (17 mg.) in phosphate buffer (17 cc.) at p_H 6·5 was oxidised with tyrosinase (2 cc.) for 25 mins. The red solution was decolorised at room temperature for 3 days and then assayed. Inactivation to the extent of 50 % had been effected and reduction of the decolorised solution did not increase the residual activity.

The results of these experiments with the free bases clearly indicate that β-oxidation in the side-chain occurs only when the terminal carboxyl group is present.

Oxidations with silver oxide.

It has already been established [Dulière and Raper, 1930] that dopa on oxidation with silver oxide yields the same indole derivatives as with tyrosinase. Preliminary experiments showed that adrenalone is oxidised by silver oxide and therefore it appeared of interest to ascertain whether dopa or *N*-methyldopa gives rise to a physiologically active base under these conditions.

An aqueous solution of the substrate (10 cc. of 0·1 %) was treated with freshly prepared silver oxide (approximately 0·05 g.) and vigorously shaken until a good red colour had developed. With dopa and *N*-methyldopa the solutions rapidly became bright red and then reddish-brown within 15 mins. The ketone gave rise to a more brownish solution. A drop of 10 % acetic acid and sodium sulphate solution (0·1 cc. of 10 %) were now added and the mixture was shaken to assist in coagulation of colloidal silver. After a few moments the latter was removed, after which the filtrate was heated in an atmosphere of hydrogen until the red tint was abolished. The true red solutions from dopa and its *N*-methyl derivative became colourless, whereas the more brownish solution from adrenalone only partially decolorised. On heating there was deposited more colloidal silver, which was removed previous to injection and hydrogenation. It was found that adrenalone was completely inactivated on oxidation in this manner. The decolorised solution from *N*-methyldopa produced a typical adrenaline-like rise in blood pressure equivalent to the latter in a concentration of 1 in 40 millions. Catalytic hydrogenation revealed no alteration in the nature or extent of the response. With dopa a similar rise in blood pressure of the same magnitude was obtained and again reduction of the decolorised solution did not cause any increased activity (see Fig. 3). With silver oxide the ketones are rapidly oxidised and consequently no accumulation takes place as with tyrosinase. The pressor activity is almost certainly due to the

presence in very small amounts of adrenaline and *nor*adrenaline respectively. As both these substances are rapidly oxidised by silver oxide, their existence is a transient one only.

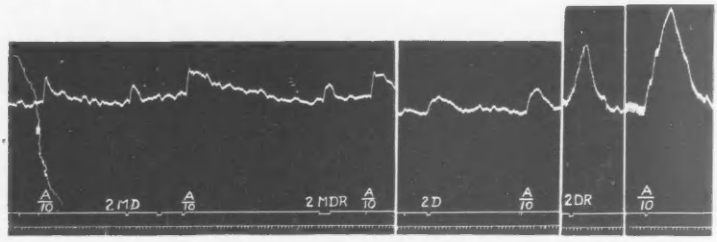


Fig. 3. Oxidations with silver oxide.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 D—2 cc. of decolorised solution from dopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.
2 DR—2 cc. of reduced decolorised solution from dopa.

On injection of the decolorised solutions from dopa and its *N*-methyl derivative, typical adrenaline-like pressor responses are obtained. Reduction of the solutions results in no enhanced pressor activity or alteration in the nature of the response. In the absence of the corresponding ketones, the rises in blood pressure are almost certainly due to the presence of small amounts of *nor*adrenaline and adrenaline respectively. N.B. The apparent increased activity of the decolorised dopa solution on reduction is due to increased sensitiveness of the animal as shown by the enhanced response to adrenaline given subsequently.

Oxidations with iron and hydrogen peroxide.

It is well known that hydrogen peroxide brings about oxidations of fundamental biological importance, and that ferrous salts, like peroxidases, may activate hydrogen peroxide. Dakin [1908] has shown that normal fatty acids and their phenyl derivatives may be oxidised to the corresponding β-ketonic acids by hydrogen peroxide. Similarly it appeared probable that the same agent might bring about β-oxidation of *N*-methyldopa and give rise to an active base by loss of CO_2. The behaviour of adrenalone, dopa and *N*-methyldopa was investigated.

An aqueous solution of the substrate (10 cc. of 0·1 %) was placed in a Thunberg tube and ferrous sulphate solution (0·2 cc. of 0·1 %) and the desired amount of a solution of hydrogen peroxide (1 %) were added. The amount of peroxide supplied was varied from 1 to 4 molecules per molecule of substrate. Oxidation was allowed to proceed in an inert atmosphere. Hydrogen was passed through the solution and expelled by way of the side arm of the Thunberg tube and a water-trap to the atmosphere. The solutions became deep reddish-brown within 10 mins. and on remaining under hydrogen for 24 hours the reddish tint was abolished. If oxidation is carried out in the presence of atmospheric oxygen, production of a black melanin-like pigment takes place. As with silver oxide, oxidation with hydrogen peroxide and iron inactivated adrenalone to the extent of 80 %. Injection of the solutions from dopa and *N*-methyldopa caused a typical adrenaline-like rise in blood pressure which was not altered on catalytic hydrogenation. The pressor response from *N*-methyldopa was equivalent to that given by 1 in 30 millions adrenaline and that from dopa to 1 in 40 millions adrenaline. The yield was found to remain constant regardless of the amount of hydrogen peroxide supplied during the oxidation.

Oxidations with peroxidase and hydrogen peroxide.

Peroxidase was prepared from horseradish as described by Bach and Chodat [1903]. The enzyme obtained in this manner is less active than the purified preparations recently described by Willstätter and Stoll [1918], but the former method does not involve the use of baryta, the last traces of which are difficult to remove, and consequently yields a product more suitable for injection. Catalase was obtained from beef-liver by aqueous extraction and alcohol precipitation.

The desired quantity of peroxidase was transferred to a dry Thunberg tube of 30 cc. capacity and dissolved in the recorded amount of phosphate buffer at p_H 6·5. Solutions of the substrate and hydrogen peroxide (1 %) were now added and air was immediately expelled by passing hydrogen through the apparatus in the manner described in the above section. The oxidation of adrenalone, dopa and methyldopa was investigated under the various conditions indicated in Table I. Oxidation was allowed to proceed until an optimum red coloration developed, when a trace of catalase was added to decompose any excess hydrogen peroxide. An evolution of gas on addition of catalase always occurred when more than two atomic proportions of hydrogen peroxide were employed. The Thunberg tube was then evacuated and filled with hydrogen several times. With each evacuation the solution was vigorously shaken to expel last traces of oxygen, and finally kept under hydrogen until decolorised.

Table I.

No.	Substrate and its amount mg.	Water cc.	Buffer p_H 6·5 cc.	Hydrogen peroxide: 1 % solution cc.	Hydrogen peroxide: Molecules per molecule of substrate	Peroxidase mg.	Time mins.
1	Dopa (5)	2·5	2·0	0·35	4·0	50	60
2	Dopa (10)	6·0	4·0	0·20	1·15	200	60
3	Dopa (5)	2·5	2·0	0·10	1·15	200	60
4	*N*-Methyldopa (10)	6·0	4·0	0·20	1·2	200	35
5	*N*-Methyldopa (10)	6·0	4·0	0·35	2·2	150	45
6	*N*-Methyldopa (5)	2·5	2·0	0·35	4·4	100	20
7	Adrenalone (2·5)	5·0	5·0	0·08	2·2	40	45

A good red colour was produced from the methylated amino-acid within a few moments and on standing under hydrogen no pigment formation took place. Using the same concentration of peroxidase it was not possible to obtain from dopa a true red colour at any stage of the oxidation (Exps. 1 and 2). After one hour the solution was dull brownish and later deposited a considerable amount of melanin. By increasing the amount of enzyme employed (Exp. 3), a more reddish solution developed, but again on standing pigment formation could not be avoided in spite of extra precautions to exclude oxygen. It is possible that in the presence of peroxidase the quinone from dopa is capable of bringing about oxidation of the indole derivative to melanin. Obviously the internal oxidation-reduction process by which the 3:4-quinone of phenylalanine is converted into the corresponding 5:6-dihydroindole derivative, and the oxidation of the latter to melanin, proceed more rapidly in the presence of peroxidase and peroxide than with tyrosinase and atmospheric oxygen or atmospheric oxygen alone. On addition of adrenalone to peroxidase, a pink coloration, which did not increase in intensity, developed immediately. No laccase was present in the peroxidase preparation as shown by the inability of the enzyme

to oxidise guaiacol in the absence of peroxide. This pink coloration is conceivably the result of a union of enzyme and substrate. *N*-Dimethylaminoacetocatechol exhibited the same phenomenon. On addition of peroxide to adrenalone and peroxidase, oxidation proceeded rapidly with the development of a dark reddish-brown solution. The reddish tint was abolished on standing under hydrogen for 24 hours. Injection of the decolorised solution showed that the ketone was inactivated to the extent of 90 %. A control solution of peroxidase and catalase alone gave no pressor response. Injection of the solutions from *N*-methyldopa caused a rise in blood pressure equivalent to that given by 1 in 2 millions adrenaline. The yield of pressor base was not affected appreciably by varying the amount of enzyme or peroxide employed for the oxidation. Reduction of the decolorised solution resulted in no enhanced pressor activity although an alteration in the nature of the response was effected (see Fig. 4).

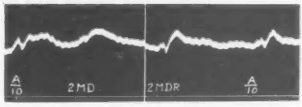


Fig. 4. Oxidations with peroxidase and hydrogen peroxide.

A/10—1 cc. of 1 in a million adrenaline.
2 MD—2 cc. of decolorised solution from *N*-methyldopa.
2 MDR—2 cc. of reduced decolorised solution from *N*-methyldopa.

Reduction of the decolorised solution from *N*-methyldopa results in no enhanced pressor activity. The response becomes more typically adrenaline-like in nature; this indicates the presence of both adrenalone and adrenaline in the original non-reduced solution.

The rise in blood pressure became more rapid and less prolonged, closely approximating to that caused by adrenaline. Since adrenalone is oxidised by peroxidase and hydrogen peroxide, it is unlikely that an accumulation of any appreciable amount of the ketone would occur, but this change in the nature of the pressor response after reduction certainly suggests that a small amount is present. On reduction of adrenalone in a concentration of approximately 1 in 90,000, a similar alteration of the pressor response without any additional rise in blood pressure takes place. With dopa (Exp. 3) the decolorised solution exhibited only a very slight, if any, pressor activity. The amount of red substance present at any given stage in the oxidation of dopa is small and consequently the formation of a pressor base, which presumably occurs simultaneously with the intramolecular change which the quinone undergoes during decoloration, is limited.

Respirometer experiments.

These were carried out in a modified form of Haldane's blood-gas apparatus in the manner previously described [Dulière and Raper, 1930]. In order to eliminate errors due to inequality in temperature, the blood-gas apparatus as well as the conical flasks and connecting tubings were immersed in a water-bath designed for this purpose. The bath was electrically heated and maintained at a temperature of 20·5°, ± 0·2°.

Solutions used. All substrates (5 mg.) except adrenaline were dissolved in water (5 cc.) and phosphate buffer (5 cc.) at p_H 6·5. The reaction flask contained 4 cc. of this solution and a further 2 cc. of buffer. With adrenaline the free base (5 mg.) was dissolved in dilute hydrochloric acid (5 cc. of 0·05 %) and 2 cc. of this solution were added to 4 cc. of buffer in the reaction vessel. In each instance

tyrosinase (1·4 cc.), which had previously been dialysed for 2 days against phosphate buffer (p_H 8), was employed. Phenylurethane was added as an antiseptic. Readings were taken periodically until oxygen uptake finally ceased and were corrected for barometric pressure and temperature. They are expressed in terms of the number of atoms of oxygen absorbed per molecule of substrate (Table II).

Table II.

Substrate (2 mg.)	No. of exps.	O_2 absorbed at N.T.P. cc.	O_2 uptake. Atoms per molecule of substrate (average)
Dopa	2	0·426–0·430	3·87
N-Methyldopa	2	0·428–0·430	4·04
Adrenaline	1	0·607	4·95
Adrenalone	1	0	0

The methylated substrate is seen to utilise more oxygen than dopa but as this difference is not great the figures suggest that the main reaction proceeding is the same as that with tyrosine. One experiment with *N*-methyldopa was carried out in the presence of glycine (0·03 %). Wiltshire [1931] has shown that amino-acids delay the oxidation of adrenaline in very dilute solution, but the addition of glycine did not affect the total oxygen uptake or rate of oxidation in our experiments. Adrenaline utilised 5 atoms of oxygen, which is in agreement with the figure of 5·3 previously recorded using undialysed tyrosinase [Dulière and Raper, 1930]. It is known that certain *ortho*-quinones are capable of bringing about oxidative deamination of amino-acids [Happold and Raper, 1925] and recently Kisch [1931] has shown that the quinone of adrenaline is particularly active in this respect. It was thought that the high oxygen uptake of adrenaline observed by Dulière and Raper might have been due to the oxidation of extraneous amino-acids in the enzyme preparation and consequently their experiment was repeated using dialysed tyrosinase.

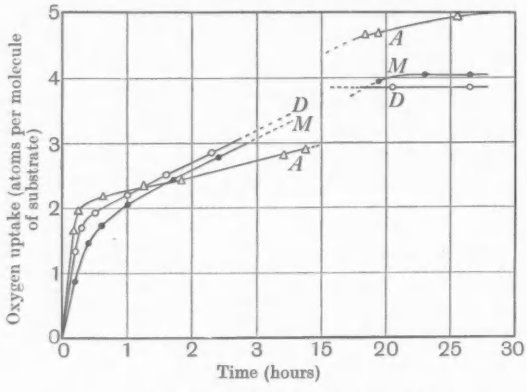


Fig. 5. *A* = Adrenaline; *M* = *N*-methyldopa; *D* = dopa.

Consideration of the rate of oxidation in these experiments (Fig. 5) shows that 2 atoms of oxygen per molecule of substrate are rapidly taken up within the first hour after which oxidation proceeds much more slowly. This is in

accordance with the view that dopa requires 2 atoms to convert it into 5:6-dihydroxyindole and a further 2 atoms to convert this into melanin. Methylation of the N atom diminishes the velocity of both stages of the oxidation; *i.e.* the speed of the two internal oxidation-reduction processes, which both the 3:4-quinone of phenylalanine and the 5:6-quinone of the dihydroindole derivative undergo, is diminished. With adrenaline the initial stage of the oxidation takes place much more rapidly than with dopa, whereas the reaction velocity of the second stage is considerably less. In the absence of the carboxyl group it is known that the speed of the 2nd intramolecular change is greatly diminished [Dulière and Raper, 1930].

Perfusion of the adrenal gland.

The technique adopted in the perfusion of this organ was essentially that described by Schkawera and Kusnetzow [1923]. The glands of a cow, together with a large piece of surrounding fatty tissue which encased 6 or 8 inches of both the aorta and vena cava, were obtained from the abattoir directly after the slaughtering of the animal and conveyed to the laboratory in an ice-bath. The blood vessels were then carefully dissected from the surrounding tissue and cannulae inserted in the adrenal arteries. In some cases it was possible to locate a main vessel leading directly from the aorta to the gland, whilst in others it was necessary to trace smaller branches back from the organ until these united and formed a vessel large enough to accommodate the cannula. The vena cava was opened, exposing the adrenal vein or veins, and the preparation set up in the usual liver perfusion apparatus. The opened flap of the vena cava was placed over the drain tube of a shallow bakelite basin and the whole preparation was contained in a metal box, the temperature of which was maintained at 37°. The perfusing fluid, warmed to 37°, was fed by gravity through the organ at a pressure of 1 metre. The perfusion was in operation within 3 to 4 hours after the death of the animal. On completion of each experiment, methylene blue was injected to determine the extent of perfusion. In most instances this represented about 10 to 20 % of total gland tissue. It is possible that only branches of the two main adrenal arteries described by Schkawera and Kusnetzow [1923] were perfused, although occasionally there appeared to be more than two independent arterial systems irrigating the gland. Methylene blue does not stain the tissues immediately; a freshly exposed surface remains pale for some minutes, after which time the medulla becomes blue, followed after a longer interval by the cortex. Conceivably the dye is reduced to the leuco-form by the reducing systems of the gland.

Oxygenated Ringer-Locke solution was perfused for 4 hours and the pressor activity of each quarter-hour fraction estimated. All fractions gave the same response (1 in 200,000 adrenaline). On re-perfusion, the activity summed proportionally and the rate of perfusion decreased. The latter phenomenon may be ascribed to constriction of blood vessels by the active substance of the perfusate. Thus, Ringer-Locke solution (210 cc.) required 17 mins. to pass through the gland in the first instance (conc. = 1 in 200,000 adrenaline), 21 mins. in the second instance (1 in 100,000) and, when perfused the third time, required 35 mins. On attempting to obtain a still more potent perfusate, the vessels were constricted so much as to render maintenance of temperature of the entering fluid almost impossible. When assayed colorimetrically by the method of Folin, Cannon and Denis [1913] the perfusate showed a greater concentration of adrenaline than that found by injection. This indicates the presence of catechol substance which is either physiologically inactive or less active than

adrenaline. The remarkable stability of the active substance of the perfusate, as described by Schkawera and Kusnetzow [1923], was confirmed. The alkaline perfusate retained its pressor activity when kept in an ice-chest for many days and was not inactivated on boiling (10 mins.). Since no known sympathomimetic base other than a ketone of the adrenalone type resists oxidation to an extent even approaching this, the perfusate (10 cc.) was reduced by catalytic hydrogenation, but no increase in pressor activity developed. The "adrenaline-like" substance of the perfusate was slowly oxidised by tyrosinase and by hydrogen peroxide when activated with either peroxidase or a trace of ferrous salt. It gave rise to a red solution with no subsequent deposition of black pigment. With silver oxide, a deep yellow solution was obtained. It was found that the perfusate was capable of delaying the oxidation of adrenaline for a long period of time. Adrenaline hydrochloride (1·2 mg.) was added to the perfusate (60 cc.), which was then incubated at 37° for 4 hours. The decrease in activity of this solution was very slight whereas a control containing adrenaline in Ringer-Locke solution was completely inactivated. On standing for 5 days at room temperature the activity of the former fell to a value equal to that of the perfusate alone which itself had decreased in activity by 50 %. Thus, added adrenaline is remarkably stabilised although it is more readily destroyed than the "adrenaline-like" substance originally present in the perfusate. In view of these facts it is more probable that this "adrenaline-like" substance is in reality stabilised adrenaline and not a stable precursor of the hormone as concluded by Schkawera and Kusnetzow [1923].

N-Methyldopa (100 mg.) in Ringer-Locke solution (100 cc.) was twice perfused through the gland, but no increase in pressor activity above that shown by a control perfusate could be demonstrated. The substrate was however remarkably stabilised on perfusion. In alkaline Ringer-Locke solution *N*-methyldopa rapidly undergoes autoxidation and the solution becomes deep brownish-black in 30 mins. The perfusate only slightly darkened overnight and did not become black until 3 days had elapsed. In the presence of tyrosinase no formation of the usual red quinone took place and only a trace of black pigment was deposited overnight. With dopa a similar stabilisation took place on perfusion. In the introduction to this communication, the manner in which this stabilisation may be effected has been discussed. Adrenalone in Ringer-Locke solution (150 cc. of 0·1 %) was perfused, but no evidence indicating reduction of the ketone by the reducing systems of the gland was obtained.

Summary.

1. As a result of the action of tyrosinase, 3:4-dihydroxyphenyl-*N*-methylalanine (*N*-methyldopa) yields a small amount of pressor base, the activity of which increases very greatly on reduction. It has been concluded that this pressor base is the ketone adrenalone which is not oxidised by the enzyme. Similarly 3:4-dihydroxyphenylalanine (dopa) yields a smaller amount of pressor substance, presumably aminoacetocatechol.

2. Production of a pressor base takes place as the result of a secondary oxidation of the β-carbon atom in the side-chain, which occurs simultaneously with the main series of reactions concerned with the formation of indole derivatives and subsequently of melanin from tyrosine. Methylation of the nitrogen atom diminishes the reaction velocity of the intramolecular change by which the indole derivative is produced and promotes β-oxidation in the side-chain.

3. Side-chain oxidation occurs only when the terminal carboxyl group is retained.

4. The above substrates, on oxidation with silver oxide and with iron and hydrogen peroxide, yield pressor bases which are not increased in activity on reduction. These are most likely adrenaline and *nor*adrenaline respectively. The corresponding ketones are also oxidised by these agents. On oxidation of *N*-methyldopa with peroxidase and hydrogen peroxide, the evidence suggests that both adrenaline and adrenalone are produced.

5. *N*-Methyldopa does not give rise to adrenaline on perfusion through the surviving adrenal gland but on the contrary is stabilised against subsequent oxidation. In the same manner, the oxidation of dopa and of adrenaline is delayed. The possible significance of this stabilisation and the synthesis of adrenaline *in vivo* are discussed.

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VII. THE SYNTHESIS OF *dl*-3:4-DIHYDROXY-PHENYL-*N*-METHYLALANINE.

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It has recently been shown by Dulière and Raper [1930] that *N*-methyltyrosine, on oxidation with tyrosinase, yields a small amount of a pressor substance. This is of particular interest in view of the close relationship between *N*-methyltyrosine and adrenaline. By oxidation and loss of CO_2 adrenaline might conceivably be produced. The first stage of this oxidation, which is in full accord with what is already known of the action of tyrosinase, involves the introduction of a second hydroxyl group in the *ortho*-position, giving rise to 3 : 4-dihydroxyphenyl-*N*-methylalanine (*N*-methyldopa). It therefore seemed desirable to synthesise this intermediate compound, as the advantages of the use of an *ortho*-dihydric substrate are manifold; notably the protracted time interval which elapses during the first stage of the oxidation is eliminated and the behaviour of the substrate with catechol oxidases and mild oxidising agents is opened to investigation.

The usual methods applied to the synthesis of aromatic amino-acids are not applicable to the synthesis of methyldopa without considerable modification. The amino-nitrogen must be free for methylation, which excludes the use of condensation products of hippuric acid, and both aromatic hydroxyl groups must be protected in order to prevent autoxidation and to avoid the complication of an unnecessary number of methylation products. In the first instance, the hydantoin method, which has been applied to the synthesis of *N*-methyltyrosine by Johnson and Nicolet [1912], was investigated. It was proposed to hydrolyse 1 : 3-dimethylpiperonylhydantoin with baryta to 3 : 4-methylenedioxyphenyl-*N*-methylalanine which would yield methyldopa on treatment with hydriodic acid. The methylation of piperonylhydantoin was attempted with both methyl iodide and methyl sulphate but only very small amounts of the dimethyl-derivative were obtained. Complete methylation of the unsaturated piperonalhydantoin is readily accomplished but in this case reduction of the corresponding dimethyl-derivative cannot be effected with sodium amalgam. Hydrolysis with baryta shatters the molecule at the double bond.

Attention was then directed to the acetylglycine method of synthesis of aromatic amino-acids recently suggested by Dakin [1929]. Piperonal is condensed with acetylglycine in the presence of acetic anhydride and sodium acetate. The resulting azlactone on mild alkaline hydrolysis yields piperonalacetaminoacetic acid, which is readily methylated with methyl sulphate. Treatment of piperonal-*N*-methylacetaminoacetic acid with hydriodic acid in the presence of red phosphorus effects a reduction of the double bond and simultaneous hydrolytic removal of the acetyl- and methylenedioxy-groups with the ultimate production of *N*-methyldopa. In order to minimise oxidation this operation is carried out

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in an inert atmosphere. The substitution for the constant-boiling hydriodic acid of equal volumes of the latter and of acetic anhydride, as employed by Harington and McCartney [1927] in the synthesis of dopa, greatly increases the yield of the final product which is further enhanced by the addition of iodine.

Efforts were made to effect a condensation of piperonal and derivatives of *N*-methylglycine, as such a product would yield *N*-methyldopa directly on treatment with hydriodic acid. The acetyl-, benzoyl- and benzenesulphonyl-derivatives of sarcosine cannot be condensed with piperonal. The respective reagents were heated on the water-bath or boiled on the oil-bath for several hours, but in each case piperonal and the sarcosine derivatives were recovered unaltered. Sarcosine itself behaves in the same manner. The corresponding unmethylated derivatives were therefore investigated, and it was found that acetylglycine and hippuric acid readily condense whereas benzenesulphonylglycine does not. The formation of an azlactone as an initial condensation product appears to be essential, and for ring formation the system —CH_2—NH—CO—R is necessary, *i.e.* the possibility of enolisation to —CH_2—N=C(OH)—R.

Experimental.

Acetylglycine was prepared by the method of Dakin [1929].

Azlactone of piperonalacetaminoacetic acid. An improved yield of this azlactone was obtained by a modification of the method of Dakin [1929]. An intimate mixture of piperonal (12·8 g.), acetylglycine (10 g.), and freshly fused sodium acetate (14 g.) was treated with freshly distilled acetic anhydride (30 cc.) and the whole heated on the water-bath for 7 hours, when the hot reaction mixture was poured into boiling water (500 cc.). On cooling, the crude azlactone was collected and washed with small quantities of alcohol and ether to remove oily impurities; M.P. 170–175°; yield 12·6 g. Crystallisation from benzene or toluene yielded the pure product which separated in bright yellow prisms, M.P. 182°.

Piperonalacetaminoacetic acid. Hydrolysis was effected as described by Dakin [1929]. The crude azlactone (9·6 g.) was dissolved in 3 molecular proportions of warm sodium hydroxide solution (0·5 *N*) and cooled gradually. The mixture was filtered and acidified with dilute hydrochloric acid, and the crude piperonalacetaminoacetic acid was collected and twice recrystallised from dilute acetic acid (10 %); M.P. 119–120°; yield 8 g.

Piperonal-N-methylacetaminoacetic acid. The acid described above (5 g.) in two molecular proportions of sodium hydroxide solution (10 %) was treated with methyl sulphate added in small portions (0·2 cc.) at regular intervals. The solution was vigorously shaken throughout the operation, which was continued until the liquid became acid to litmus, and methylated products began to separate. A third molecular proportion of sodium hydroxide was then added and methylation continued until in all 4 cc. of methyl sulphate had been utilised. Maximum yields were obtained when the methylation had been extended over a period of 3 to 4 hours at room temperature. The oily precipitate obtained on acidification consisted of a mixture of unchanged piperonalacetaminoacetic acid and its *N*-methyl-derivative, together with hydrolytic products resulting from the sodium hydroxide treatment. Separation of the former was effected by the use of chloroform and acetone. The methylated product was found to be soluble in chloroform and but slightly soluble in acetone, whilst the unmethylated acid is soluble in acetone and insoluble in chloroform. The reaction solution was therefore directly transferred from the methylation bottle into a large separating funnel, acidified with dilute hydrochloric acid, and

extracted with chloroform. The residue collected by filtration was returned to the separating funnel and extraction was continued until the crude piperonal-*N*-methylacetaminoacetic acid was completely removed. On removal of the solvent by distillation, the residue was taken up in hot dilute acetone (20 %), boiled for a few moments with a trace of animal charcoal, and filtered. On cooling the pure product separated in well-defined colourless plates, M.P. 196°; yield 2·5 g. A further 0·25 g. was recovered from the acetone filtrate by concentration, chloroform extraction, and crystallisation from acetone.

Piperonal-*N*-methylacetaminoacetic acid is very soluble in warm organic solvents and slightly soluble in cold acetone, ether, and alcohol. It is readily dissolved by cold alkalis and by dilute mineral acids on warming. Crystallisation takes place readily from dilute acetone, alcohol, or acetic acid.

(Found: C, 58·6; H, 4·93; N, 5·42 %. $C_{13}H_{13}O_5N$ requires: C, 59·3; H, 4·94; N, 5·33 %.)

Crystallisation of the residues insoluble in chloroform from dilute acetic acid (10 %) gave unchanged piperonalacetaminoacetic acid (1 g.).

dl-*3 : 4-Dihydroxyphenyl*-N-*methylalanine.* Piperonal-*N*-methylacetaminoacetic acid (5 g.), red phosphorus (5 g.), and resublimed iodine (5 g.) were suspended in a mixture of hydriodic acid (25 cc.; sp. gr. 1·7) and acetic anhydride (25 cc.) contained in a small round-bottomed flask which was fitted by means of a ground-glass joint to the reflux condenser. The system was evacuated and filled with CO_2 by means of a capillary tube led through the condenser. A small stream of gas was then passed continuously and expelled through a water trap to the atmosphere. The mixture was boiled on the sand-bath for 7 hours, cooled under CO_2, and filtered. The phosphorus and iodine residues were washed with 50 % acetic acid (50 cc.) and the combined filtrates added to an excess of lead acetate solution (400 cc. of 20 %). Lead iodide was removed, and washed several times by shaking with water (200 cc.). It was found impossible completely to free the lead iodide from catechol substances as repeated washings always gave an intense green coloration with ferric chloride. The filtrate and wash-waters were made slightly alkaline with dilute ammonia, which brought down the lead salt of *N*-methyldopa as a white flocculent precipitate. The latter was collected, washed, and decomposed in aqueous suspension (250 cc.) with hydrogen sulphide. Lead sulphide was removed, washed, and the combined filtrates were immediately concentrated under diminished pressure, CO_2 being led into the capillary of the Claisen flask. The concentrate (25–50 cc.) was cautiously neutralised to Congo red with a few drops of dilute ammonia (0·1 %) and allowed to crystallise *in vacuo* over sulphuric acid until the mother-liquor attained the bulk of 2–3 cc. The crude product was collected, washed with a few drops of water, and redissolved in the minimum quantity of boiling water containing a drop of sulphurous acid which minimises oxidation. On cooling no crystallisation took place. The solution was therefore neutralised to Congo red and allowed to crystallise *in vacuo*. After two such recrystallisations *N*-methyldopa was obtained in colourless clusters or sheaves of radiating needles which were usually pigmented; M.P. 275°; yield 1·5 g. The use of charcoal as a decolorising agent resulted in the oxidation of appreciable amounts of the product. For analysis the sample was recrystallised four times, M.P. 280°, but still contained traces of a light brown pigment.

(Found: C, 56·8; H, 6·19; N, 6·59 %. $C_{10}H_{13}O_4N$ requires: C, 56·8; H, 6·16; N, 6·63 %.)

The melting-point was not constant but was found to vary considerably with the rate of heating, in which respect the substance behaves in the same manner

as *N*-methyltyrosine. The values recorded above are those taken under like conditions; the rate of heating was controlled by means of a manometer in the gas circuit. *N*-Methyldopa is readily soluble in hot water, sparingly so in cold water, and insoluble in organic solvents. Unlike dopa the methylamino-acid does not crystallise well from water. In neutral aqueous solution with ferric chloride it gives an intense green coloration which passes into reddish-purple on addition of very dilute ammonia. In aqueous solution, autoxidation, with the development of a pink coloration, proceeds more rapidly than with dopa.

The above investigation has been carried out under the supervision of Prof. H. S. Raper, to whom the writer wishes to acknowledge his indebtedness.

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VIII. EXPERIMENTS ON NUTRITION.

XII. COMPARATIVE VITAMIN B_1 VALUES OF ANIMAL FOODSTUFFS.

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Cooper [1912; 1914] first examined animal tissues for their vitamin B (= B_1) values: 20 g. muscle, 5 g. heart, 12 g. brain, 3 g. liver, 35 g. milk and 3 g. egg-yolk prevented polyneuritis in pigeons over a period of 50 days. Smaller amounts would prevent loss of weight. Eddy [1916] showed that extracts of pancreas produced growth of vitamin B-deficient rats. Chick and Hume [1917] found that an alcoholic extract of the hard roe of turbot corresponding to 35 g. of tissue was effective in curing polyneuritis in pigeons. Osborne and Mendel [1917; 1918] determined that 19 % of dried heart, 22 % of kidney, 23 % of liver, 32·5 % of brain produced normal growth of rats. Osborne, Mendel and Cannon [1923] found that 1·5 g. of dried egg-yolk was effective for growth of rats. Sherman and Smith [1931] quote experiments with pigeons by Brodie, who found that kidney was not so good as liver and heart, and that lung and spleen had little vitamin B_1. Hoagland [1929] compared dried lean beef and lean pork; the latter was richer. Macy, Outhouse, Graham and Long [1927] ascertained that 20 to 25 cc. of cow's milk, and 25 to 30 cc. of human milk sufficed for normal growth of rats. Chick and Roscoe [1929] found that 1 g. of dried = 3·9 g. of fresh egg-yolk produced a weekly increase of 10 to 14 g. in young rats.

Though the methods of examination have been rather variable, the results of the above investigations are in fair agreement. It is difficult to make a list of the comparative values of the various tissues. To complete our experiments on the comparative vitamin B_1 values of foodstuffs [1929; 1931] a large number of animal tissues have been tested by the same method, and the results show some distinct differences from those of previous workers.

Experimental.

Pairs of pigeons were used in the tests, housed and fed in the same way as in our previous experiments.

Owing to the high water content of the animal foods, the various tissues were minced and heated in a basin on a water-bath until they were sufficiently dry to mix with white flour and make into pills. Cooked in this way, the tissues were more palatable to the pigeons, and there was not any refusal by the birds to eat the mixture of high protein content, except in the cases of brain and soft roe of fish, but the food was eaten when the birds were hungry. Refusal to eat only occurred during the periods of onset of polyneuritis. Lack of appetite is the first symptom of insufficient vitamin B_1. The first experiments with egg-

Table I.

Date	Time in weeks	Diet: Egg-yolk	Diet: White rice	Diet: $Ca(OH)_2$	Weights, g.: Cock No. 42	Weights, g.: Hen Nos. 25, 35	Remarks
3. iii. 26					445	370	
to		50	50	0·5			
28. vii. 26	21				405	325	Hen laid 8 eggs; 1 hatched and died; others deserted
to							
15. ix. 26	7				425	205	Hen not well and was chloroformed. Organs anaemic. Cock quite well
					No. 1	No. 2	
5. i. 26					520	660	
to		33	67	0·5			
24. ii. 26	7				325	405	Hen paralysed and showed head retraction. Both lost weight. Cock not well
			White flour	Salts	No. 81	No. 96	
4. ix. 29					400	325	
to		50	49	1			
16. x. 29	6				285	330	Cock showed head retraction and died
					No. 346		
23. x. 29					455	340	New cock
to							
25. xii. 29	16				385	285	Hen showed head retraction and was cured
to							
5. ii. 30	15				265	—	Cock showed head retraction and was cured
					No. 56	No. 14	
19. vi. 29					425	400	
to		40	59	1			
31. vii. 29	6				330	330	Hen showed head retraction and died
to							
7. viii. 29	7				300	—	Cock showed head retraction and was cured
					No. 47	No. 94	
10. vii. 29					405	410	
to		30	69	1			
14. viii. 29	5				305	290	Hen showed head retraction and was cured
to							
11. ix. 29	9				225	—	Cock paralysed and died
					No. 56	No. 94	
9. x. 29					405	420	
to		60	39	1			
18. xii. 29	10				420	385	Hen showed head retraction and died
						No. 96	
8. i. 30					435	360	
to							
19. iii. 30	23 (9)				430	340	Hen died from worms
						No. 13	
26. iii. 30					—	320	
to							
21. v. 30	32 (8)				345	305	Cock showed head retraction and was cured
to							
6. viii. 30	(25)				—	335	Hen ate very little
					No. 85	No. 44	
4. ii. 31					550	460	
to		70	29	1			
19. viii. 31	28				515	420	Hen laid 7 eggs; deserted. Both quite well

Table I (*contd.*).

Date	Time in weeks	Diet: Egg-yolk	Diet: White flour	Diet: Salts	Weights, g.: Cock No. 62	Weights, g.: Hen No. 79	Remarks
18. ii. 31					390	400	
to		60	39	1			
16. ix. 31	30				370	285	Hen laid 4 eggs. Not well from 17th week. Died from cyst in abdomen
		Ox-liver			No. 78	No. 10	
8. i. 30					375	410	
to		30	69	1			
5. iii. 30	8				300	370	Hen showed head retraction and died
to							
2. iv. 30	12				245	—	Cock showed head retraction and died
					No. 28	No. 347	
19. ii. 30					345	440	
to		50	49	1			
26. iii. 30	5				300	455	Cock showed head retraction and died
to							
16. iv. 30	8				—	410	Hen died
					No. 40		
9. iv. 30					410	—	
to							
2. vii. 30	12				225	—	Died. Did not eat for 3 weeks
					No. 4	No. 6	
15. x. 30					435	455	
to		60	39	1			
20. v. 31	31				435	460	Hen was not well from 7th to 15th week, but recovered and laid 4 eggs; deserted
					No. 90	No. 35	
18. iii. 31					380	385	
to		50	49	1			
27. v. 31	10				365	305	Hen showed head retraction and was cured
to							
2. xii. 31	37				415	450	Remained well and laid 10 eggs; all deserted
		Sheep's kidney			No. 65	No. 58	
17. xii. 30					415	405	
to		60	39	1			
17. vi. 31	26				395	345	Hen laid 10 eggs, all deserted. Lost weight from 11th week. Both quite well
					No. 92	No. 91	
1. iv. 31					400	380	
to		50	49	1			
14. x. 31	28				390	380	Hen laid 14 eggs, all deserted. Both quite well
					No. 92	No. 91	
4. xi. 31					420	395	
to		40	59	1			Hen laid 2 eggs
30. xii. 31	8				420	330	Hen showed head retraction and died
to							
20. i. 32	11				350	—	Cock showed head retraction and died
		Sheep's heart			No. 65	No. 58	
15. i. 30					395	370	
to		30	69	1			
26. ii. 30	6				375	340	Both showed head retraction and were cured

Table I (*contd.*).

Date	Time in weeks	Diet: Sheep's heart	Diet: White flour	Diet: Salts	Weights, g.: Cock No. 65	Weights, g.: Hen No. 99	Remarks
23. ix. 31					375	420	
to		60	39	1			
30. iii. 32	27				400	445	Both quite well
					No. 100	No. 1557	
2. xii. 31					455	390	
to		70	29	1			
6. iv. 32	18				445	415	Both quite well
					No. 85	No. 44	
13. i. 32					600	455	
to		50	49	1			
20. vii. 32	27				500	410	Hen laid 10 eggs; all deserted. Cock lost weight from 5th to 15th week, then remained constant
					No. 18	No. 93	
2. iii. 32					530	420	
to		40	59	1			
17. viii. 32	24				485	275	Hen lost weight at 15th week. Showed head retraction and died
to							
19. x. 32	33				495	—	Cock quite well
		Sheep's brain					
					No. 342	No. 33	
8. i. 30					540	390	
to		30	69	1			
5. ii. 30	4				400	335	Cock showed head retraction and was cured
to							
19. ii. 30	6				—	295	Hen was paralysed and died
					No. 31	No. 32	
17. ix. 30					415	440	
to		60	39	1			
19. xi. 30	9				285	325	Cock showed head retraction and died. Hen was paralysed and died
					No. 23	No. 93	
23. ix. 31					385	410	
to		80	19	1			
4. xi. 31	6				230	295	Cock showed head retraction and died. Hen was paralysed and died
		Sheep's lungs					
					No. 17	No. 29	
10. vi. 31					345	330	
to		60	39	1			
5. viii. 31	8				265	230	Both showed head retraction and were cured
					No. 18	No. 39	
28. x. 31					505	470	
to		80	19	1			
16. xii. 31	7				410	310	Both showed head retraction. Cock was cured, hen died
		Tripe					
					No. 32	No. 58	
16. ix. 31					415	310	
to		60	39	1			
21. x. 31	5				215	260	Both showed head retraction. Cock died, hen was cured
					No. 85	No. 44	
21. x. 31					540	430	
to		80	19	1			
18. xi. 31	4				410	365	Both showed head retraction and were cured

Table I (*contd.*).

Date	Time in weeks	Diet: Pancreas	Diet: White flour	Diet: Salts	Weights, g.: Cock No. 1156	Weights, g.: Hen No. 99	Remarks
13. v. 31					435	430	
to		50	49	1			
8. vii. 31	8				280	325	Both showed head retraction. Cock died, hen was cured
					No. 100	No. 1557	
29. vii. 31					405	390	
to		70	29	1			
16. ix. 31	7				290	230	Both showed head retraction and were cured
		Beefsteak			No. 29	No. 58	
10. ii. 32					435	385	
to		50	49	1			
23. iii. 32	6				255	230	Hen showed head retraction and died
to							
30. iii. 32	7				235	—	Cock showed head retraction and died
					No. 90	No. 35	
16. iii. 32					380	415	
to		80	19	1			
18. v. 32	9				400	235	Hen was paralysed and died
to							
1. vi. 32	11				290	—	Cock showed head retraction and was cured
		Dried skimmed milk			No. 236	No. 304	
18. ii. 31					540	465	
to		50	49	1			
16. ix. 31	30				525	445	Hen laid 5 eggs, all deserted. Both well
					No. 40	No. 3	
3. vi. 31					355	355	
to		40	59	1			
6. i. 32	31				455	440	Both well, but increased in weight
					No. 236	No. 304	
23. ix. 31					515	470	
to		30	69	1			
9. xii. 31	11				500	460	Cock showed head retraction and died
to							
13. i. 32	16				—	390	Hen was paralysed and died
					No. 17	No. 37	
25. ii. 31					445	430	
to		20	79	1			
6. i. 32	6				320	270	Hen was paralysed and died
to							
27. i. 32	9				280	—	Cock was paralysed and died
		Soft roes (herring)			No. 65	No. 58	
13. xi. 29					405	375	
to		30	69	1			
18. xii. 29	5				345	295	Both showed head retraction and were cured
					No. 336	No. 341	
23. x. 29					480	360	
to		50	49	1			
6. xi. 29	3				445	335	Cock died
to					No. 342		
13. xi. 29	4				495	—	Hen died
to							
11. xii. 29	4				375	—	Cock showed head retraction and was cured

Table I (*contd.*).

Date	Time in weeks	Diet: Hard roes (cod)	Diet: White flour	Diet: Salts	Weights, g.: Cock No. 1156	Weights, g.: Hen No. 35	Remarks
11. xii. 29					430	380	
to		50	49	1			
18. vi. 30	27				410	390	Hen laid 6 eggs: 2 hatched. Squabs died
					No. 72	No. 5	
1. i. 30					350	390	
to		30	69	1			
16. vii. 30	28				465	415	Hen laid 8 eggs; all deserted
					No. 100	No. 1557	
31. xii. 30					435	405	
to		30	69	1			
8. vii. 31	27				400	395	Hen laid 12 eggs, all deserted. Lost weight at 19th week but recovered
					No. 72	No. 5	
17. ix. 30					340	385	
to		20	79	1			
5. xi. 30	7				275	280	Hen showed head retraction
to							and died
10. xii. 30	12				280	—	Cock paralysed and died
					No. 56	No. 98	
5. xi. 30					455	370	
to		20	79	1			
3. xii. 30	4				385	330	Hen died
to							
24. xii. 30	7				245	—	Cock showed head retraction and died
		Fish muscle (cod)					
					No. 8	No. 20	
16. iii. 32					405	415	
to		80	19	1			
13. iv. 32	4				320	285	Cock showed head retraction
to							and died
20. iv. 32	5				—	300	Hen showed head retraction and was cured
		Mussels			No. 345	No. 347	
4. xii. 29					395	445	
to		50	49	1			
1. i. 30	4				185	215	Cock showed head retraction
to							and died
15. i. 30	6				—	270	Hen showed head retraction and was cured

yolk were made many years ago; 1 % of slaked lime was mixed with the yolk to supply calcium salts, and the mixture was poured over white rice and allowed to dry. In the later experiments the yolk was removed from hard-boiled eggs and mixed with the flour. In these and the other tests a salt mixture corresponding to the ash of fish meal (the standard protein used in our previous experiments) was added as source of mineral matter. Cod-liver oil as source of vitamins A and D was omitted as animal tissues contain these vitamins. Dried skimmed milk was used in preference to whole fresh milk, since vitamin B_1 is known to be in the watery part and not in the cream. In the case of mussels the whole animals were cooked in a little water, the shells removed and the water and tissue evaporated until of suitable consistency. It was difficult to

decide the proportion at which to make the first trial; the earlier work indicated a level of 30 %, but it was found that this was seldom enough and 50 % was first tried. The results of the experiments are given in Table I.

Discussion.

Many of the results of these experiments were unexpected from the work of previous authors. Egg-yolk was believed to be rich in vitamin B_1. Our first experiments in 1926 showed clearly that 33 % of yolk in the diet was not enough, but that 50 % sufficed. On repeating the tests in 1929 with yolks from hard-boiled eggs, 30, 40 and also 50 % were insufficient. The first trial with 60 % yolk did not give a clear result, and a repetition in 1931 showed that this quantity was just enough. 70 % was necessary to maintain birds in good health. 60 % must be regarded as the minimum. Liver has been regarded as one of the best sources of vitamin B_1. Neither 30 nor 50 % in the diet supplied enough vitamin B_1. The minimum quantity was 60 %. Kidney was expected to be less rich than liver; in this case 50 % was required to maintain the pigeons for 26 weeks. Heart was found to be good by Cooper. The pigeons were maintained on 60, 70 and 50 %; 40 % was not quite enough. Eddy's test with a preparation from pancreas indicated that this organ was a rich source of

Table II.

	Water %	Percentage amount in diet		Maintenance	Comparative vitamin B_1 value	
		Wet	Dry		Wet	Dry
Dried yeast	0	4	4	+	100	100
Marmite	27	6	4·5	+	67	90
Cereals	11	40	35	+	10	12
Middlings	12	10	9	+	40	44
Bran	10	20	18	+	20	22
Wheat germ ("Bemax")	6	6–7	6·5	+	63	61
Malt extract	22	40	31	+	10	13
Nuts	4	20, 40	19, 38	+	20, 40	22, 44
Pulses	12	30	26	+	13	15
Coconut	—	—	—	—	—	—
Orange juice	87	70	23	+	6	20
Tomatoes	93·5	80	21	+	5	20
Other fruits	—	—	—	—	—	—
Cabbage	88	80	32	+	5	13
Watercress	94	80	20	+	5	20
Artichokes	80	80	45	+	5	9
Leeks	85	80	37	+	5	10
Parsnips	73	60	35	+	7	12
Potatoes	81	90	64	+	4	7
Other vegetables	—	—	—	—	—	—
Egg-yolk	47	60	32	+	7	13
Liver	71	60	17	+	7	23
Kidney	76	50	12	+	8	33
Heart	78	50	11	+	8	36
Lung	77	—	—	—	—	—
Brain	78	—	—	—	—	—
Tripe	83	—	—	—	—	—
Pancreas	29	—	—	—	—	—
Muscle	69	—	—	—	—	—
Dried skimmed milk	5	40	38	+	10	11
Hard roes	79	30	6	+	13	62
Soft roes	69	—	—	—	—	—
Fish muscle	81	—	—	—	—	—
Mussels	84	—	—	—	—	—

vitamin B_1, but even with 70 % in the diet, the pigeons became polyneuritic. This may be accounted for by the large amount of fat in the pancreas we used. Our aim has been to ascertain the comparative values of foods of human consumption. Brain also gave an unexpected result. Our data show that vitamin B_1 is practically absent from brain. Lung, tripe and muscle similarly do not contain more than traces of vitamin B_1. Milk is not rich in vitamin B_1. At least 40 % of dried skimmed milk was required to maintain the pigeons. Of the fish foods, only the hard roe of cod had an appreciable amount of vitamin B_1. The hard roe of cod, and presumably of other fishes, is a very rich source of vitamin B_1. The pigeons were maintained with 30 % in the diet.

Summary.

Animal foodstuffs are variable in their content of vitamin B_1. In their fresh condition they contain relatively less than cereals and pulses, but more than fruits and vegetables. In the dried state, egg-yolk is equivalent to cereals, the other tissues exceed cereals and pulses in their vitamin B_1 contents. The data are collected in Table II, compared with the value of dried yeast, which has been taken as standard in our tests, and the mean values for cereals, pulses, fruits and vegetables, nuts, *etc.*

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IX. DEVELOPMENT OF URICASE IN TADPOLES OF *RANA TEMPORARIA*.

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(*Received December 5th, 1932.*)

Numerous papers have been published on the appearance of enzymes during the ontogenetic development of animals. In the case of uricase, however, very little is known, either of its distribution in the individual organs of adult animals or of its presence in embryos. Employing the methods described by Truszkowski [1930] it was found possible to demonstrate the existence of this enzyme in comparatively small portions of material (0·2–1 g.), and these methods have now been applied to the determination of the stage of development at which uricase makes its appearance in the frog. It was found by Truszkowski and Goldman (unpublished) that the kidneys and liver of adult frogs contain uricase, but not the eggs.

According to Herlitzka [1907] the fertilised egg contains indophenol oxidase, catalase, amylase, and invertase from the very beginning of its development, whilst tyrosinase appears on the 15th day of development. No work appears to have been done on the purinolytic enzymes in the ontogenesis of frog larvae. The experiments described below show that uricolytic action appears on the 9th day after hatching, augments till the 16th day and remains constant till the 34th day, after which it again increases.

Experimental.

The eggs were collected from a pool near Warsaw, on April 10th. They were examined with a lens, and only those masses which appeared to be at the same stage of development (morula) were taken. The eggs originated from *Rana temporaria*, and their developmental stage indicated that they had been laid 3 days before collection.

The jelly was spread in layers about 1 cm. in depth in glass and earthenware tanks in the laboratory, together with a little water, and development was allowed to proceed at room temperature (16–18°), the water being changed every other day. The tadpoles were, after the 1st week, fed with egg-yolk powder. 1 g. portions, separated from the jelly, and roughly dried with filter-paper, were taken at intervals, and triturated in a mortar with a solution of lithium urate (0·05 %; p_H 8·0), and the ground suspension was washed into a flask with further portions of urate, 20 cc. of which were taken in all. 1 cc. of toluene was added to ensure sterility and the suspensions were shaken 3–4 hours at room temperature (16–18°) and then left for a further 20–21 hours, when they were filtered, and uric acid was determined in the filtrates by the method of Folin and Denis.

Table I.

Date	Day of development	Developmental stage	mg. uric acid found in control	mg. uric acid found in system	mg. uric acid oxidised
10. iv	3	Morulae	10·2	10·2	0·0
13. iv	6	Non-motile embryos, 3 mm. long	10·2	10·2	0·0
15. iv	8	Motile, with external gills	10·2	10·2	0·0
18. iv	11	,, 1·0 cm. long	10·2	10·2	0·0
20. iv	13	,, 1·4 cm. long	10·2	10·2	0·0
22. iv	15	Gills disappear	10·2	9·6	0·6
25. iv	18	—	10·8	10·2	0·6
27. iv	20	—	10·4	8·2	2·2
29. iv	22	—	10·2	7·2	3·0
2. v	25	—	10·4	7·2	3·2
4. v	27	—	10·4	7·2	3·2
6. v	29	—	9·6	6·8	2·8
10. v	33	Hind-limbs appear	8·6	6·0	2·6
17. v	40	—	8·8	5·6	3·2
23. v	46	Fore-limbs appear	10·4	1·0	9·4

The results are given in Table I. The first sign of uricolytic action appears on the 15th day of incubation, and coincides with retraction of the gills. From the 20th to the 40th day uricolytic activity remains fairly constant, an abrupt rise being noticed with the development of the fore-limbs and the disappearance of the tail on the 46th day.

Discussion.

The appearance of uricase coincides with the retraction of the external gills of the tadpole, and the abrupt augmentation in uricolytic power observed on the 46th day with the appearance of the fore-limbs. In view of the observation that both the kidneys and the livers of adult frogs contain uricase, it may be that these two stages represent development of uricase in the kidneys and liver respectively; the small size of the animals rendered the experimental verification of this supposition impossible. According to Fürbringer [1878] the renal function is performed in *R. temporaria* larvae by the pronephros, the first rudiments of the mesonephros, characteristic of the adult animal, not appearing until the larvae attain a length of 16 mm. The abrupt rise in uricolytic action coincident with metamorphosis might thus be ascribed to the development of the mesonephros; should this be the case, uricolytic action observed before the appearance of the fore-limbs and after the retraction of the gills must be due to hepatic uricase. Since, however, the liver functions as such long before retraction of gills takes place (Konopacki and Konopacka [1926] found that deposition of glycogen began on the 9th day of development), it is difficult to explain why hepatic uricase did not make its appearance at an earlier stage, since no striking histological or functional changes take place in this organ during the entire period of larval development. It is possible that changes in the circulation consequent on retraction of the gills might in some way be connected with the appearance of uricase on the 15th day of development. On the other hand, Herlitzka's observations [1907] indicate that the liver is not initially possessed of its full adult equipment of enzymes; in this case tyrosinase makes its appearance at the same time as uricase, *viz.* on the 15th day.

SUMMARY.

1. Uricase is absent from *Rana temporaria* eggs.
2. It makes its appearance in the larvae on the 15th day of development.
3. The uricase content remains practically constant from the 19th to the 46th day, when it again rises abruptly.
4. The first period of uricolytic activity is ascribed to hepatic uricase and the second to the development of the mesonephros.

The authors wish to express their gratitude to Prof. St J. Przyłęcki for his interest in the above research.

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X. THE INFLUENCE OF THE BASAL DIET IN THE DETERMINATION OF VITAMIN A.

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(*Received December 8th, 1932.*)

In estimating the potency of preparations containing vitamin A by growth tests on rats, the large variation among the animals has for a long time presented difficulty. Recent contributions towards the improvement of the position are the issue of carotene as a standard for vitamin A by the Permanent Commission on Biological Standardisation of the League of Nations [Report, 1931], and the statistical treatment of results by Coward *et al.* in their recent publications.

The minimum effective dose is still frequently quoted as a criterion of activity, although there are grounds for believing that this value may be a function of the basal diet used, or may change with other variable factors such as the strain or nurture of the rats employed. The only recently published papers on variations in the basal diet are that of Coward, Key and Morgan [1929], who found with their diet that caseinogen after being extracted and heated to render it free from vitamin A did not permit of satisfactory growth, even when supplemented with large doses of cod-liver oil, and that of Honeywell, Dutcher and Ely [1931], who described the effects of different yeasts upon the incidence of xerophthalmia. Coward *et al.* [1929] provisionally ascribed their results to the absence from treated caseinogen of a new dietary factor present in "light white casein" and in other substances, whilst Honeywell *et al.* suggested that yeast contained either some vitamin A or a factor concerned only with the prevention of xerophthalmia.

The experiments described below were carried out in order to explain some anomalies which arose in the course of the routine testing of certain concentrates for vitamin A. It has been found not only that the basal diet exerts a great influence upon the size of the dose of vitamin A necessary to prevent decline and death, but also upon the observed symptoms of continued avitaminosis.

For some time past we have used for the routine testing of vitamin A concentrates a diet (A 5) of the following composition:

Diet A 5.

Extracted and heated caseinogen	20
Rice starch	60
Marmite	5
Salts [Drummond and Watson, 1922]	5
Hardened fat	9·9
Radiostol (3000 U)	0·1

Young rats put on to A 5 diet at about 4 weeks old grow well at first, cease growing after 6–10 weeks and decline more or less rapidly. Either just before they cease growing or at the same time, they show definite signs of xerophthalmia, which gets progressively worse until almost invariably they become blind before death. If they are given a sufficient dose of cod-liver oil or vitamin A concentrate at the time when growth has ceased, their health is quickly restored and they continue to grow and live indefinitely in perfect condition (Fig. 1). Females regularly become pregnant and give birth to their young, which do not however survive for more than 2–3 days while the mother remains upon the synthetic diet.

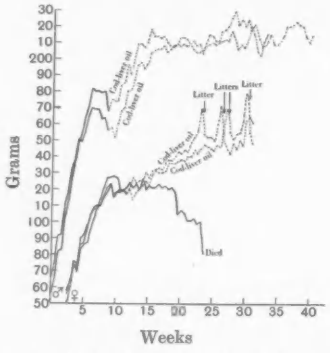

Fig. 1. Growth curves for a litter of rats fed on diet A 5. When they ceased growing four were given daily doses of 0·02 cc. of a cod-liver oil giving a Carr and Price colour test of 10. One female acted as negative control and received no supplement of vitamin A.

After young rats have ceased growing on A 5, their growth and health can be restored by a daily dose of 0·02 cc. of cod-liver oil (giving a value of 10 by the Carr and Price [1926] colour test); 0·01 cc. diluted to 0·02 cc. with arachis oil gives a reasonable but slower growth rate, and these are the standard doses we usually employ. Coward *et al.* [1931], using a different basal diet, reported fairly good growth with most samples of cod-liver oil tested by them in a dose of 1 mg. and sometimes even less per day. Coward (private communication) regards 1 mg. per day as a suitable standard dose for a cod-liver oil giving a value of 10 by the Carr and Price colour test. Moore [1930] has also found that 0·002 mg. of carotene dissolved in arachis oil will restore the growth of rats depleted of their vitamin A stores upon yet another type of diet, but with A 5 such a dose is without effect. Collison *et al.* [1929] found 0·003 mg. effective when given in hardened cottonseed oil. Using still another basal diet it is stated by Olcovich and Mattill [1931] that 0·005 mg. of carotene per day in ethyl laurate or cottonseed oil was always "sufficient to cause marked increase in weight and recovery from ophthalmia." Nelson and Jones [1928], using a basal diet containing caseinogen extracted 10 times with hot 95 % alcohol, quote 10 mg. of cod-liver oil in 0·2 cc. of arachis oil as an amount that will cause resumption of growth when no decline has already started. It is thus apparent that there is a wide diversity in the amount of vitamin A-containing substances necessary to restore growth on different basal diets.

Since a statistical method developed by Coward has gained a position of considerable importance, it appeared to be essential to compare her diet with that employed by us (A 5), which we have found to be eminently satisfactory

for the purpose of vitamin A tests. Coward's diet (A 51 C) has the following composition:

Diet A 51 C.

"Light white casein"	15
Rice starch	73
Dried yeast	8
Salts (Steenbock No. 40)	4

2 units of vitamin D are given twice a week by pipette.

The principal differences between diets A 5 and A 51 C are as follows.

1. A 5 contains 20 % of heated and extracted caseinogen, whilst the protein constituent of A 51 C is 15 % of "light white casein."

2. A 5 contains 10 % of added fat, whilst A 51 C has none other than that contained in the "light white casein" and yeast.

3. The vitamin B requirements in A 51 C are supplied by 8 % of dried yeast, whilst A 5 contains 5 % of marmite.

4. A 51 C contains dextrinised starch, whilst in A 5 the starch is not cooked at all.

5. Different salt mixtures are employed, 5 % in A 5 as compared with 4 % in A 51 C.

6. A 5 provides about 30 units per day of vitamin D to a rat consuming 10 g. of diet, whilst with A 51 C only 2 units of vitamin D are given per week.

Rats maintained on diet A 5 invariably develop xerophthalmia and require large doses of substances containing vitamin A for cure and restoration of growth, whilst rats on A 51 C rarely suffer from xerophthalmia, although they cease growing in about the same time, and require very much smaller amounts of vitamin A for restoration of growth. To explain these differences two alternatives of the utmost importance must be considered.

1. Xerophthalmia may be caused by a simple vitamin A deficiency.

2. There is some other factor concerned in conjunction with vitamin A in the prevention of xerophthalmia.

If 1 be true it follows that A 51 C and other similar diets contain some vitamin A, insufficient for the maintenance of growth, but enough to prevent the occurrence of the disease xerophthalmia. It would thus follow that smaller doses of vitamin A should be required to bring the total up to that which can supply the needs of the animals.

If 2 be true, the additional factor must be present in some constituent of A 51 C but entirely absent from A 5. It must be present in cod-liver oil and other vitamin A concentrates, because almost perfect health and growth can be obtained with A 5 when supplemented by one of these. The need for larger doses of supplements with A 5 than with A 51 C would be explained by the animal's need of the additional factor.

The work that follows is a comparison of the two diets having such different effects and was undertaken in the attempt to find out what ingredients of A 51 C provide either the additional amount of vitamin A, or some other substance which in its absence can protect rats against xerophthalmia.

We cannot claim yet to have made a complete comparison, but the whole subject appears to be of such importance in the assay of samples of vitamin A as to warrant a publication of the work which has been done and the conclusions which have been reached up to the present.

Experimental.

The experiments were planned in order to observe the effect of changing each constituent of the diet separately. The different diets used are summarised in Table I. Vitamin D was given as radiostol solution incorporated in the diet. The amount, in units per 100 g. of diet, is shown in the last column. Supplements of vitamin A, when given, were always diluted so that the required dose was contained in 0·02 cc. of arachis oil.

Young rats were placed upon the experimental diet when about 4 weeks old, having reached a weight of not less than 40 g. They were weighed twice a week and given small doses of cod-liver oil or vitamin A concentrate when they had reached a constant or declining weight, the time of onset of xerophthalmia or any other easily detected symptom being noted. At least one animal from every litter was left upon the basal diet alone.

Direct comparison of diet A 51 C with diet A 5.

When a group of rats which had ceased growing on diet A 5 were given supplements (0·02 cc.) of vitamin A in oils diluted to a blue colour of 0·5, and failed to resume growth, a change to diet A 51 C caused an immediate increase in weight. Unfortunately, a negative control grew just as well as the rats on oils, and the experiment was therefore useless from the point of view of the assay of the oils (Fig. 2). The rat that lived longest had a slight recurrence of

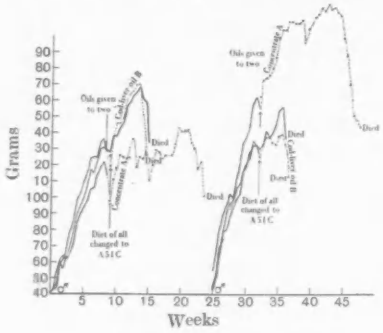

Fig. 2.

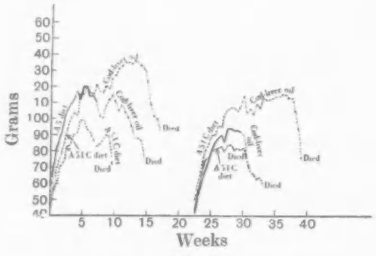

Fig. 3.

Fig. 2. Growth curves for rats from two litters. All were given diet A 5 alone until they ceased growing. Those of the first litter were then given daily doses of 0·02 cc. of cod-liver oil (Oil A) and a concentrate (Oil B) diluted to give a colour value of 0·5 blue in the Carr and Price test. When they made no response the diet of all was changed to A 51 C, and two of the second litter were given oils as well.

Fig. 3. Comparison of the growth curves of rats (one litter) fed on diet A 5 (solid line) and A 51 C (thick dotted line). When they ceased growing all were given a daily dose of 0·02 cc. of cod-liver oil diluted to give a Carr and Price test of 1·0.

the secretion from the eyes, but the other two had perfectly clear eyes. All of them, however, suffered from an inflammatory condition of the tail, ears and nose.

By the kindness of Dr Coward we were enabled to repeat this experiment using some of the actual diet made up in the Pharmaceutical Society's laboratories. The growth curves for this litter are shown in Fig. 3. This time the rats

were given the diet from the beginning of the experiment, half the litter being kept on diet A 5 for the sake of comparison. It is clear from the curves that diet A 51 C supplemented by small doses of cod-liver oil was able to support life for much longer than diet A 5. None of the three animals developed xerophthalmia, whereas two on A 5 became blind and the third also had a definite secretion round the eyes. On the other hand the animals on A 51 C had an inflammatory condition of the ears, tails and legs, but their litter-mates on A 5 had no skin lesions at all.

The effects of the various constituents of the diets upon growth and the development of xerophthalmia were then tested separately (see Table I).

Table I.

No. of diet	Heated and extracted caseinogen	"Light white casein"	Rice starch	Rice starch dextrinised	Marmite	Dried yeast	Salts (Drummond and Watson)	Salts (Steenbock No. 40)	Hardened oil	Radiostol 3000 U/cc.	Units of vitamin D
A 5	20	—	60	—	5	—	5	—	9·9	0·1	300
A 51 C	—	15	—	73	—	8	—	4	—	—	3
A 51	—	20	60	—	5	—	5	—	9·9	0·1	300
A 50 F	20	—	60	—	5	—	5	—	—	0·1	300
A 510 F	—	20	60	—	5	—	5	—	—	0·1	300
A 5 Y	20	—	60	—	—	5	5	—	9·9	0·1	300
A 58 Y	20	—	60	—	—	8	5	—	9·9	0·1	300
A 51 CM	—	15	—	73	8	—	—	4	—	—	3
A 5 DS	20	—	—	60	5	—	5	—	9·9	0·1	300
A 5 YDS	20	—	—	60	—	5	5	—	9·9	0·1	300
A 51 DS	—	20	—	60	5	—	5	—	9·9	0·1	300
A 51 OR	—	20	60	—	5	—	5	—	9·9	—	—
A 50 R	20	—	60	—	5	—	5	—	9·9	—	—
A 50 FY	20	—	60	—	—	5	5	—	—	0·1	300
A 510 FY	—	20	60	—	—	5	5	—	—	0·1	300
A 50 FY 8	20	—	60	—	—	8	5	—	—	0·1	300

"Light white casein."

When "light white casein" replaces the heated and extracted caseinogen of diet A 5, xerophthalmia is not prevented but its onset is delayed, and the resulting condition is usually less severe. The obvious conclusion from this observation is that "light white casein" contains vitamin A, but not sufficient to enable the rat to grow and thrive without further supplement. By a rough calculation from an experiment (not reported here) the amount of vitamin A was shown to be about 1/200 of the amount contained in cod-liver oil, on the assumptions (1) that the type of caseinogen is the only difference responsible for the different results obtained with the two diets, and (2) that the individual rats all eat about the same quantity, say 10 g. per day.

The amount of butter-fat in "light white casein" varies between 0·6 and 4·5 % (the highest and lowest values obtained in 29 estimations for which I am indebted to Mr T. T. Cocking) with an average value of 2·5 %. Taking the vitamin A content of butter fat as 1/15 that of good cod-liver oil [see Nelson and Jones, 1928] a rat eating 10 g. of diet can receive the equivalent of about 3 mg. cod-liver oil per day.

A direct comparison of the two caseinogens has been made measuring the amount of vitamin A in "light white casein" in terms of carotene. Observations were recorded of the time taken to develop xerophthalmia on diet A 5 supplemented with different doses of carotene and on diet A 5 made with "light white casein" instead of extracted and heated caseinogen (diet A 51). The time until the onset of xerophthalmia was used as the criterion of the test.

In Fig. 4 the results are shown diagrammatically. Each horizontal line represents the results from one litter. It is clearly demonstrated that "light white casein" and carotene both retard the onset of xerophthalmia. Taking into account the number of rats on each diet, we have:

								No. of rats
Litter	3.	A 51	is more effective	than	0·01	mg.	carotene	8
,,	4.	,,	,,	,,	0·01	,,	,,	4
,,	5.	,,	,,	,,	0·01	,,	,,	10
,,	6.	,,	,,	,,	0·002	,,	,,	6
,,	7.	,,	less	,,	0·05	,,	,,	8
,,	8.	,,	,,	,,	0·002	,,	,,	6
,,	9.	,,	more	,,	0·05	,,	,,	6
,,	11.	,,	,,	,,	0·002	,,	,,	10
,,	12.	,,	,,	,,	0·01	,,	,,	
		,,	,,	,,	0·002	,,	,,	8
		,,	less	,,	0·05	,,	,,	
,,	13.	,,	,,	,,	0·01	,,	,,	
		,,	,,	,,	0·002	,,	,,	8
		,,	,,	,,	0·05	,,	,,	

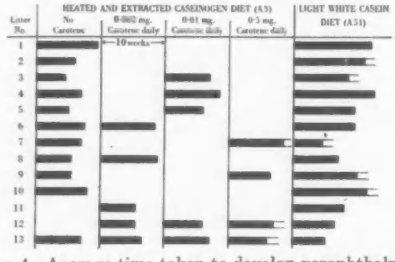

Fig. 4. Average time taken to develop xerophthalmia.
N.B. Dotted lines indicate that one animal or more did not develop xerophthalmia at all.

The conclusion seems fairly clear that 20 % of "light white casein" in our diet is equivalent to, or slightly less than, 0·05 mg. daily of carotene in its power of protection against xerophthalmia.

From growth tests with diet A 5 we know that 0·05 mg. carotene (in arachis oil) is about as effective as 10 mg. cod-liver oil (in arachis oil), which gives the ratio 1 : 200 for the vitamin A content of "light white casein" and cod-liver oil. The ratio agrees with the previous calculation.

To illustrate the regularity with which xerophthalmia appears in members of the same litter, Table II shows the time to its onset in the individual members of the two largest litters in the test.

Table II. *Number of weeks to the onset of xerophthalmia.*

		In arachis oil		
	No carotene	Diet A 5 0·002 mg. carotene	0·01 mg. carotene	Diet A 51 ("light white casein")
Litter 5	5	—	5·5	8·0
	—	—	6·0	8·0
	—	—	5·5	8·0
	—	—	6·5	11·5
	—	—	5·5	11·0
Litter 11	—	5·5	—	7·5
	—	5·0	—	7·5
	—	4·5	—	8·0
	—	6·0	—	7·5
	—	6·0	—	7·5
	—	4·5	—	9·0

The difference in severity of the xerophthalmia developed on the two diets is shown in Table III.

Table III.

	Diet A 5 %	Diet A 51 %
Completely blind	53	2
One eye blind	12	5
Severe xerophthalmia in both eyes	18	10
Xerophthalmia in both eyes	18	31
Xerophthalmia in one eye	—	31
Eyes clear	—	21

The most usual picture on the "light white casein" diet (A 51) is a maintenance of weight over a fairly long period of time with an almost regular recurrence of "secretion" from the eyes, sometimes accompanied by "snuffles." Occasionally rats on A 51 died rather suddenly without any obvious cause. Those on the diet containing extracted and heated caseinogen (A 5) developed sore eyes either before they stopped growing, or after a decline in weight had commenced. They often remained alive for 5 to 10 weeks afterwards, losing weight throughout that time.

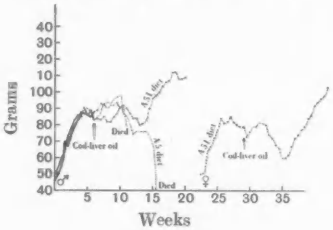

Fig. 5.

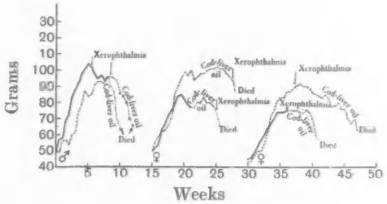

Fig. 6.

Fig. 5. Growth curves of a litter of 4 rats. Two were fed on diet A 5 (solid line) and two on diet A 51 (thick broken line). When growth ceased all were given a daily dose of 0·02 cc. of cod-liver oil diluted to a blue figure of 1·0 by the Carr and Price test.

Fig. 6. Growth curves of a litter of 6 rats. Three were fed on diet A 5 (solid line) and three on diet A 51 (thick broken line). When growth had ceased all were given a daily dose of 0·02 cc. of cod-liver oil diluted to a blue figure of 1·0 by the Carr and Price test. The arrows show the points at which definite xerophthalmia developed.

The growth curves are not very instructive because the rats were not kept on the diets long enough for differences to show, but on the whole the animals on the largest dose of carotene grew better than those on "light white casein," which in turn grew better than those on the heated and extracted caseinogen.

Fig. 5 shows that 0·02 cc. daily of cod-liver oil diluted to a value of 1·0 blue unit by the Carr and Price test may be sufficient to maintain life and even stimulate growth when given with a diet containing "light white casein" (A 51), but it is not sufficient when combined with a diet containing extracted and heated caseinogen (A 5).

Further confirmation that "light white casein" contains vitamin A is afforded by the growth curves of the rats given in Fig. 6. In each case xerophthalmia developed about $2\frac{1}{2}$ weeks later in the rats on diet A 51 than in those on A 5, and their lives were prolonged to about the same extent. Xerophthalmia was never severe in the three animals on A 51 diet whereas the three on A 5 were blind.

Fat.

A striking difference between the two diets is the inclusion of 10 % of fat in A 5 and its consequent higher calorific value. Fig. 7 gives the growth curves for a litter of rats on diet A 5 from which the fat was removed when growth had ceased (diet A 50 F). The caseinogen was also changed at the same time (diet A 510 F) in the diet of half the animals. As they still did not grow they were given small supplements of oils; a concentrate of 550 blue value diluted 1 part in 1000, and cod-liver oil diluted 1 part in 20. In neither case can the growth that ensued be attributed to the dose of oils, for the two animals that were given no oil started to grow also. All members of the first group had severe xerophthalmia (two became blind), whilst those of the group on "light white casein" had much less severe xerophthalmia, but dermatitis appeared on the ears, nose and body. The skin lesions were ultimately cured by replacing the fat in the diet. It is evident that the "light white casein" was responsible for the decrease in severity of xerophthalmia, and that the removal of fat had no effect upon its onset.

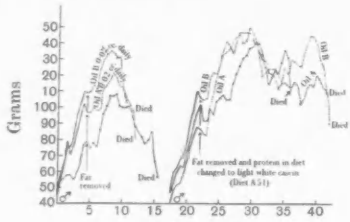

Fig. 7.

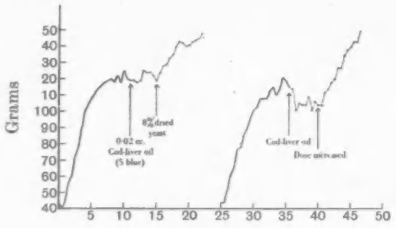

Fig. 8.

Fig. 7. Growth curves of rats from one litter showing the effect of the removal of fat from the diet. The second group which was given "light white casein" instead of extracted and heated caseinogen survived for a longer time.

Fig. 8. Recovery of growth of two rats on diet A 5 (1) by giving 8 % dried yeast instead of 5 % marmite, (2) by increasing the dose of cod-liver oil.

It is exceedingly probable that the removal of fat caused a partial deficiency of vitamin B, which would be responsible for the skin lesions. Evans and Lepkovsky [1929] state that even with 8 % dried yeast the addition of fat is still beneficial.

Yeast.

When 5 % of marmite was replaced by 5 % of yeast (diet A 50 FY) in the fat-free diet (A 50 F), the response of the rats to small doses of vitamin A concentrate and cod-liver oil was not improved. An increase to 8 % dried yeast (diet A 50 FY 8) appeared to lessen the severity of the resulting xerophthalmia. This is in accordance with the results of Honeywell, Dutcher and Ely [1931], who found variations between 20 and 90 % in the incidence of xerophthalmia on their diet when using different yeasts; they regarded this as evidence that yeast contains some vitamin A. Hume and Smith [1928] have also commented on the greater resistance to xerophthalmia shown by rats receiving an increased marmite ration.

When growth has ceased from partial deprivation of vitamin A, it may occasionally be stimulated again by increasing or by changing the source of vitamin B. As an example, Fig. 8 shows the growth curve of a rat "run out"

on diet A 5 and then given 0·02 cc. daily of cod-liver oil diluted 1 part in 2 with arachis oil. As it still failed to grow, the 5 % of marmite in the diet was changed to 8 % of dried yeast (diet A 58 Y), after which the eyes recovered and growth was resumed, whilst the negative control (not shown) whose diet was not changed, became blind and died.

The second curve (Fig. 8) shows a similar experiment in which, instead of changing the source of vitamin B, the dose of cod-liver oil was increased with the expected result that the eyes recovered and growth was resumed.

Thus exactly the same effect may be produced by increase in the dose of vitamin A or by alteration of the source of vitamin B. The most probable explanation is that dried yeast contains some vitamin A. The additional amount, together with the small dose of cod-liver oil was sufficient, but with the concentrate not sufficient, to support growth or cure xerophthalmia.

When the dried yeast of diet A 51 C was replaced by marmite (diet A 51 CM) we found no difference in the rates of growth when small doses of vitamin A were given, but all developed a mild condition of xerophthalmia in addition to inflammation of the ears, nose and skin, which occurred on all fat-free diets. This also is in accordance with the results of Honeywell, Dutcher and Ely [1931].

Starch.

Dextrinised starch in place of uncooked starch (diet A 5 DS) was not effective in preventing xerophthalmia or in promoting growth upon small doses of oils. The growth curves of a litter of rats fed on diet A 5 DS are given in Fig. 9. The negative control became blind after 10 weeks on the diet and died. Another

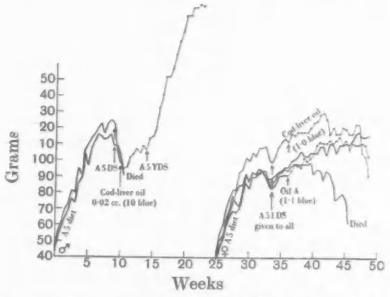

Fig. 9. Growth curves of rats on diets A 5 DS, A 5 YDS and A 51 DS, with and without cod-liver oil.

had severe xerophthalmia which was cured by a large dose of cod-liver oil, but growth was only resumed when the marmite was exchanged for dried yeast (diet A 5 YDS). This change also effected a cure of the severe dermatitis from which the animal was suffering. When "light white casein" replaced the heated and extracted caseinogen of the diet (A 51 DS), the animals survived for a longer time whether the starch was dextrinised or uncooked, and there was no difference that could be attributed to the small dose of oils given to two of them.

Vitamin D.

In the course of an experiment on the toxicity of a particular sample of irradiated ergosterol, a litter of six rats was divided into two groups, one of which received a large excess of irradiated ergosterol (0·1 % of the diet), whilst

the other was given the same amount of pure ergosterol. At the beginning of the experiment none was given any vitamin A at all. The three on excess of vitamin D developed severe xerophthalmia, but the eyes of those receiving no vitamin D remained perfectly clear. Ergosterol was therefore tested on a number of animals to see if it could cure xerophthalmia, but, as it was not effective, the only conclusion that can be drawn is that a certain amount of vitamin D is necessary for the experimental production of xerophthalmia. This has been observed also by Nelson and Jones [1928].

When a much larger amount of vitamin D (180 U weekly) was added to diet A 51 C, only two of a litter of five developed xerophthalmia. The experiment cannot therefore be regarded as conclusive. Doses of cod-liver oil and vitamin A concentrate (diluted to a value by the Carr and Price test of 0·1) appeared to cause a rather longer survival, but the growth performance of the two receiving oils showed only a slight superiority over that of the negative control. All the rats on this test developed skin lesions, which in the case of one of the males were very severe. They were ultimately cured by a daily dose of olive oil.

A litter of rats was placed upon a diet containing "light white casein" but no radiostol (A 51 OR). "Light white casein" was used instead of extracted and heated caseinogen, in order to ensure that they had a small amount of vitamin A, but not sufficient to prevent the development of xerophthalmia. When the animals started to lose weight seriously they were given the usual 180 units per week of vitamin D, and growth was resumed for another 5–8 weeks, when they failed and died, presumably from vitamin A deficiency, but none of them suffered from xerophthalmia or showed any of the characteristic signs. It would appear then that a sufficiency of vitamin D is needed from the beginning.

It has been shown that foodstuffs rich in vitamin A can exert a considerable influence upon the effects produced by excess of irradiated ergosterol, and that deficiency of vitamin A causes the development of urinary calculi, a condition also associated with an excess of vitamin D. This suggests that there is a relation between the biological action of these two vitamins, and that there is some significance in the fact that they commonly occur together.

That both dried yeast and "light white casein" contain some vitamin D is shown by the following experiments.

After 4 weeks on a rachitogenic diet (Steenbock and Black 2965) 20 % of "light white casein" was given to two rats of a litter of six. Two received the diet containing 20 % of extracted and heated caseinogen, and one was given the rachitogenic diet with two units of standard vitamin D daily. When the rats were killed and sections of the tibiae, radii and ulnae stained with silver nitrate solution, the animals on "light white casein" and standard showed complete healing and practically perfect bones, whilst those on the heated and extracted caseinogen diet showed inferior calcification, though better than that of the negative control. The difference in the calcium and phosphorus contents of the two caseinogens is small, and we may assume that the superior calcification on the "light white casein" diet was due to vitamin D. The test indicates that it contains more than 1 unit per g.

In a second experiment, dried yeast was included in the same diet to the extent of 5 % and fed to two members of one litter, two others each receiving 2 units of standard vitamin D daily. The calcification of the bones of the rats on dried yeast was not so good as that of the two on standard, showing that dried yeast contains less than 4 units of vitamin D per g.

Condition of the rats on the different diets.

We have made careful observations of the symptoms encountered on the different basal diets. The results are summarised in Tables IV and V. Table IV contains the results for all animals on diets containing no added fats, Table V for those on the diets containing fats. The majority of the animals, as shown in the third column, were given very small supplements of the vitamin A oils when the growth curves had reached a plateau, because the object for which the experiments had been undertaken was to find a diet that was definitely deficient in vitamin A, and yet would give a growth response with these small doses. Neglecting these doses entirely as having no effect, the following facts are evident from these Tables, in which some attempt is made to indicate the severity of the various conditions by the number of positive signs.

1. Diets containing extracted and heated caseinogen in the absence of vitamin A invariably produce xerophthalmia of considerable severity.

2. Inflammatory conditions of the skin are particularly liable to occur on diets containing no fat. Under the heading "body sores" the average number of positives on fat-free diets is 1·2, on fat-containing diets 0·3.

Table IV. *Diets free from fat.*

Rat No.	Diet	Vitamin A supplement colour value	Xerophthalmia	Skin lesions: Body	Skin lesions: Ears	Time on diet in weeks
		"Light white casein" diets.				
GLA. 208	A 51 C	Oil A 0·5	+	+	+++	24
212		„ B 0·5	−	+	+++	15
213		None	−	++	++	15
G. 724		Oil A 0·5	+	++	+	23
725		„ B 0·5	+	−	−	12
726		None	+	−	−	11
GLB. 270		Oil B 1·0	−	++++	++	17
271		None	−	+	+	9
272		Oil B 1·0	−	++	++	16·5
G. 740	A 51 C, with marmite instead of dried yeast	Oil A 0·5	++	+	++++	23
742		„ B 0·5	++	+	++++	23
739		None	++	+++++	−	23
741		„	++	+++	+++	23
G. 733	A 5, with "light white casein," no fat	Oil A 0·5	+++	++++	+	23
736		„ B 0·5	++++	+++++	+++	23
737		None	+++	−	++++	19
731		Oil A 0·5	+	+++++	++	23
732		„ B 0·5	+++	+++	−	23
GLA. 210	A 5, with "light white casein," no fat, dried yeast	Oil A 11·0	+	−	+	28
211		None	−	−	++	28
214		„	+	+	+++	28
		Extracted and heated caseinogen diets.				
GLB. 15	A 5, no fat	Oil A 1·1	++++	−	−	15
19		„ B 1·0	+++	−	−	17
16		None	++	−	−	21
17		„	+	−	−	16
G. 734		Oil A 0·5	++++	−	−	12
735		„ B 0·5	++	−	−	12
738		None	+++++	−	−	16
GLB. 14		Oil A 1·1	+++	−	−	16
18		„ B 1·0	+++++	−	+	18
22		None	+++++	−	+	21
GLB. 20	A 5, 8 % dried yeast, no fat	Oil A 1·1	++++	−	++	21
21		None	−	−	+	10
23		Oil B 1·0	++	−	++	16

Table V. *Diets containing fat.*

Rat No.	Diet	Vitamin A supplement colour value	Xerophthalmia	Body sores	Sore ears	Time on diet in weeks
GLA. 219	A 5	None	++	−	+	14
GLB. 11	A 5	Oil A 1·1	++	−	+	18
GLB. 12	No radiostol	„ B 1·0	+++	−	+	17
GLB. 7	„	„ A 1·1	+	−	+	12·5
GLB. 8	„	„ B 1·0	+	−	+	11·5
GLB. 27	A 5, with "light white	„ A 1·1	+	−	++	21
28	casein"	„ B 1·0	+	−	−	21
GLB. 9	A 5, with 2 U vitamin D	—	++++	+	+	24
10	„	—	++++	+	+	22
13	„	—	+	++	+++++	24
GLB. 30	A 5, with dextrinised starch	None	Blind	−	+	11
31		Oil B 10·0	++	++++	+++	20
GLB. 24	A 5, with "light white	„ B 1·0	−	−	+	21
25	casein" and dextrinised starch	„ A 1·1	+	−	−	25
GLA. 221	A 5, yeast instead of mar-	„ A 1·1	+++++	−	−	16·5
222	mite	„ B 0·5	++++	−	−	11·5
H. 281	A 5, 8 % yeast	„ B 0·5	++++	−	+	9
282		None	+++	−	+	10
283		Oil A 5·5	+	−	+	9·5
284		„ B 1·0	+++	−	+	11
285		„ A 1·1	+++	−	+	14
FR. 200	A 5	None	+++++	−	−	10
201		„	+++++	−	−	8
202		„	+++++	−	+	11
203		„	+++++	−	−	15
204		„	+++++	−	−	14
205		„	+++	−	−	10
206		„	+++++	−	−	11
207		„	+++++	−	−	14
208	A 51	„	++	−	−	20
209		„	−	+	+	9
210		„	−	−	−	10
211		„	−	−	−	11
212		„	+	−	−	12
213		„	+	−	−	15

It is true that many of the animals showing no symptoms might have developed them had they lived long enough, for they are seldom definite in an animal maintained on the diet for less than 20 weeks, but it is noticeable that with diet A 5 the rats may be only just kept alive by small doses of vitamin A for indefinite periods, yet the fur and skin always remain healthy.

Discussion.

Since A 5 diet invariably produces xerophthalmia, it seems certain that it is devoid of vitamin A. Since it produces no observable effect that is not associated with deprivation of vitamin A, it appears equally certain that there is no other deficiency. Although the caseinogen used must suffer both physical and chemical change from the treatment that it undergoes in the destruction of the vitamin A contained in it and derived originally from the milk from which it was prepared, it still remains a perfectly good source of protein for the rat. If this were not so, we should not find good growth and even reproduction when the animals are given vitamin A as well. It is true that the mothers cannot rear their young upon this diet, but the same can be said of all synthetic diets. That they suffer no permanent impairment of health is shown by the fact that they make most satisfactory members of the breeding stock after they have been used for

vitamin A testing. For example, one such female after 6 months on a vitamin A test has produced and reared five litters comprising 6, 10, 10, 6 and 7 young respectively. This rat is in no way exceptional and shows clearly that the 6 months' feeding on our vitamin A-free diet, supplemented in this particular case by radiostoleum, has done her no harm.

The simplest explanation of the fact that xerophthalmia does not develop on most diets is that they contain ingredients which are not devoid of vitamin A. This, however, does not explain the results of Coward *et al.* [1929; 1930] on the presence in "light white casein" of some factor capable of supplementing cod-liver oil. It is possible that cod-liver oil and most concentrates contain two factors which are together associated in producing the effects commonly ascribed to vitamin A. If this is so, the proportion may be liable to vary, and the particular sample of cod-liver oil used by Coward, Key and Morgan [1929] in their experiment must have been deficient in one of them which was subsequently supplied by "light white casein," watercress, lettuce or one of the other substances found to be effective by these workers. It would be exceedingly interesting if they were to repeat their experiments using calciferol as their source of vitamin D throughout, instead of irradiating the diet, which was their method of ensuring an adequate supply of this vitamin in the original experiment with extracted and heated caseinogen. The more recent work on the generation of vitamin D shows how uncertain this method may be, and the irregularity of behaviour and slow decline of their rats is rather suggestive of a shortage of vitamin D.

There is no doubt that the solution of the whole problem of vitamin A testing would be greatly facilitated by a clearer definition of vitamin A deficiency than has so far been given. Since the promotion of growth is a property of all substances necessary for food, it is a very unsatisfactory criterion, and cessation of growth is certain to occur long before there is a complete deficiency of any specific factor. For this reason it is impossible to pronounce a diet as satisfactory for vitamin A testing simply because growth may be arrested at a certain stage and stimulated again by addition of the test substance. Where a standard is used it is not of paramount importance to have a standardised basal diet for testing the vitamin preparation, but when the desired result is the "minimum effective dose," it is obviously essential that the diet should be completely devoid of the particular vitamin. A comparison of absolute results obtained in different laboratories is rendered quite useless if this is not the case, and where the standard employed is not a pure sample of the substance measured, as in the estimation of vitamin A in terms of carotene, the necessity for a standardised diet must be emphasised.

Summary.

1. "Light white casein" and dried yeast both contain vitamin A and some vitamin D.

2. 5 % of marmite is a sufficient source of vitamin B when the diet also contains fat.

3. Skin lesions were observed on fat-free diets and were not prevented by 5 % of marmite or 8 % of dried yeast.

4. Xerophthalmia is a symptom of extreme deficiency of vitamin A and occurs regularly upon a diet which is completely devoid of the vitamin.

5. Xerophthalmia can be prevented or its onset retarded by a diet containing "light white casein," yeast or other source of vitamin A.

6. Xerophthalmia does not readily occur when there is also a deprivation of vitamin D.

7. Observation of the rate of incidence of xerophthalmia is a satisfactory method for the estimation of vitamin A.

8. The modified Drummond and Watson diet commonly used by us, which contains extracted and heated caseinogen, is deficient in vitamin A and in no other vitamin known to be necessary for the rat, and is therefore a very satisfactory basal diet for its determination.

In the course of this work I have had the advantage of frequent discussions with Dr T. Moore to whom I wish to express my thanks. I am also indebted to Dr F. H. Carr and Dr S. W. F. Underhill for constant help and encouragement, to J. Lione for his unfailing care of the experimental animals and to the Directors of the British Drug Houses for allowing me to publish this work.

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XI. THE UPTAKE OF ETHYL ALCOHOL BY THE ISOLATED TORTOISE'S HEART.

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(*Received December 9th, 1932.*)

The distribution of ethyl alcohol between the blood and the tissues is discussed fully by Winterstein [1926, pp. 225–248]. Most authors have found that alcohol is distributed fairly uniformly throughout all the tissues in the body which have a good blood supply.

Hansen [1925; Tables VI and VII] found in two rabbits, in which the blood concentration of ethyl alcohol was 0·13 and 0·30 % respectively, concentrations of alcohol in the heart of 0·12 and 0·28 %.

Carpenter [1929; see Table IV] studied the alcohol distribution in hens exposed to ethyl alcohol vapour. He found that, with blood concentrations of alcohol varying from 0·25 to 0·5 %, the concentration in the heart was about 75 % of the blood concentration.

These facts suggest that alcohol is distributed fairly uniformly between the blood and the heart, and the present authors measured the uptake of alcohol by the isolated tortoise's heart in order to determine whether the same distribution occurred when tissues were exposed to concentrations of alcohol higher than those which could occur in the intact animal.

Methods.

Isolated hearts of *Testudo graeca* were used. They weighed between 1 and 2 g. They were fitted with two cannulae, a venous one in the sinus and an arterial one in the aorta. The general arrangement of cannulae was that described by Clark and White [1928] for the frog's heart. The hearts were allowed to beat with their natural rhythm. The excursion of the ventricle was registered by an isotonic lever. The hearts did not beat well with Ringer's fluid alone and were therefore perfused with a mixture of 95 % Ringer's fluid (composition as given by Clark and White [1928]) and 5 % tortoise serum. This method involved the use of a limited volume of perfusion fluid and alcohol was lost, not only by uptake by the heart, but also by evaporation into the air. The perfusion fluid was therefore changed periodically and the alcohol content of each change of fluid was measured at the end of the perfusion. The alcohol contents of the perfusion fluids and of the hearts were measured by Southgate's method as described by Carter [1928].

Experimental results.

Vernon [1910–11] studied the action of alcohol on tortoises' hearts. He found great individual variations in sensitivity but concluded that 0·5 *M* ethyl alcohol usually produced a 50 % reduction in force, that it produced its full action in 9 minutes, after which the heart's activity remained at a constant level, and that on washing out there was almost instantaneous recovery.

We used 0·75 *M* alcohol in most experiments and found that this produced a reduction both in amplitude of contraction and of systolic pressure of between 50 and 80 %. The alcohol caused however a variation of both frequency and amplitude of contraction, and therefore the mechanical response did not give a satisfactory quantitative measure of the action. We confirmed all the conclusions of Vernon mentioned above. It is important to note that the highest concentrations of alcohol used in our experiments did not arrest the heart, and therefore the analyses refer to tissues which were still capable of functioning.

We made 13 experiments and the results are shown in Table I. The figure for the alcohol content of the fluid after perfusion refers to the last of the changes of perfusion fluid that were passed through the hearts. The figures show in the first place that the heart takes up a certain amount of alcohol in

Table I.

Content of perfusion fluid in ethyl alcohol g. per 100 cc.	Duration in min.		Ethyl alcohol content		
			A	B	
	Perfusion	Washing out	Fluid after perfusion g. per 100 cc.	Heart g. per 100 g.	Ratio $100\frac{B}{A}$
3·5	2·5	—	3·13	1·59	51
3·5	5·0	—	3·16	1·25	40
3·5	15·0	—	2·90	1·67	58
3·5	30·0	—	3·07	1·65	54
3·5	60·0	—	3·32	1·90	58
3·5	180·0	—	2·98	1·67	56
3·5	30·0	4·0	—	0·37	—
3·5	180·0	20·0	—	0·19	—
2·62	180·0	—	2·60	1·25	48
0·70	5·0	—	0·65	0·46	71
0·70	30·0	—	0·53	0·40	75
0·70	30·0	—	0·72	0·49	68
0·35	30·0	—	0·34	0·26	77

less than 5 minutes and that no more alcohol is taken up even when the perfusion is continued for as long as 3 hours. When the alcoholised heart is perfused with alcohol-free solution the alcohol is washed out rapidly and about three-quarters is removed in 4 minutes. These experiments show that the heart both takes up and releases ethyl alcohol rapidly. Pickford [1927] found that in the frog's heart ethyl alcohol would produce half its full action on the mechanical activity in less than 5 seconds. Our methods did not permit measurement of the alcohol uptake in periods of less than 2 minutes, and hence we cannot say whether the uptake of alcohol actually proceeds as rapidly as do the changes in mechanical activity, but our results as far as they go support this view. The most interesting fact shown by our figures is that the distribution of ethyl alcohol between the Ringer-serum mixture and the heart does not follow Henry's law. The six figures for 3·5 % alcohol show an average fluid/heart distribution ratio of 100/53, whereas the three figures obtained with 0·7 % alcohol show an average distribution ratio of 100/71. This result cannot be accounted for by differential solubility, but is in accordance with Warburg's theory that alcohol is adsorbed on surfaces in the heart cells.

The figures given in Table I can be fitted by the usual adsorption formula. If x and y represent the concentrations of alcohol in the fluid and heart respectively, then the results are fitted approximately by the formula $Kx^{\frac{1}{n}} = y$, when $K = 0{\cdot}64$ and $n = 1{\cdot}2$.

Extrapolation of the experimental results shows that when x falls to 0·2, y will be 0·17, and when x is 0·1, y will be 0·095. Our results therefore are

concordant with those obtained by Hansen and by Carpenter who found that with concentrations of alcohol of 0·5 % or less the concentration of alcohol in the heart was 75 % or more of the blood concentration. Our experiments show however that when the alcohol concentration is raised this ratio becomes much less.

SUMMARY.

The relation found between the concentrations of ethyl alcohol outside and inside the cells of a perfused tortoise heart shows that the uptake of alcohol does not depend on differential solubility, but resembles an adsorption process.

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XII. AN IMPROVED METHOD FOR THE MEASUREMENT OF TISSUE RESPIRATION.

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(*Received December 9th, 1932.*)

In the measurement of the respiration of surviving animal tissues it is desirable that the conditions should be as physiological as possible; hence a method which allows the use of media containing bicarbonate in equilibrium with the physiological tension of CO_2 is often needed. Of the methods hitherto available, the second method of Dickens and Šimer [1931] is the only one by which the oxygen and CO_2 of respiration can be determined under such conditions. This method is of great importance, as it enables accurate determinations of the respiratory quotient to be made on one slice of animal tissue immersed either in phosphate buffer, bicarbonate-Ringer solution or serum, the latter two being kept in equilibrium with a suitable gas mixture containing CO_2.

We have found this method to be capable of giving very good results; but it requires careful manipulation, and a somewhat elaborate calculation is necessary in order to obtain the oxygen uptake. Moreover four manometers are necessary for a single determination, namely, two with tissue for determining the bound and total CO_2 at the beginning and end of the experiment, one for determining the amount of CO_2 introduced with the gas mixture and one for use as a thermobarometer. We have introduced two modifications which have rendered possible a considerable improvement in these respects. The first is the introduction of a new type of flask, and this in turn made it possible to effect a considerable simplification by the application of the principle of compensation as used in the Barcroft differential manometer. In the method now to be described only one manometer is needed for each experiment, and the oxygen and CO_2 of respiration are obtained directly with the simplest calculation.

Principle of the method.

Two flasks of equal volume, provided with suitable means for introducing acid or alkali during the experiment, are attached to the two sides of a Barcroft differential manometer. Equal amounts of tissue and of the medium (*e.g.* bicarbonate Ringer solution or serum) are placed in both flasks, which are then filled with the appropriate gas mixture (in this case 5 % CO_2 and 95 % O_2). Since the Barcroft differential manometer is not affected by changes of temperature or of barometric pressure occurring during the experiment, the need for a separate thermobarometer is eliminated. Also, since equal amounts of CO_2 are introduced into the two flasks with the gas mixture, subsequent absorption of this will affect both sides of the manometer to the same extent[1], so that the need of a separate manometer for the analysis of the gas mixture is likewise eliminated. The procedure is therefore as follows. After filling with the gas mixture as described below, the apparatus is shaken for a few minutes in the

[1] This will be the case even if the flasks are of unequal volumes. See the paper of Dickens and Šimer.

thermostat to ensure proper equilibration, and the taps are closed. The reading should then be zero, *i.e.* the manometer should be level. The acid is then introduced into the left-hand flask, killing the tissue and liberating the bound CO_2. It is not necessary to read the apparatus at this stage, but it is shaken say for an hour, during which time the tissue in the right-hand flask continues to respire. Exactly one hour after introducing acid into the left-hand flask, acid is similarly introduced into the right-hand flask, and the apparatus is shaken for a further 15 mins. until the reading becomes steady. Then the state of affairs is exactly the same in both flasks, except that the tissue in the right-hand flask has respired for an hour longer than that in the left-hand flask. The reading of the apparatus is then taken. This reading, which may be called h_1, corresponds to the difference between the oxygen absorbed and the CO_2 evolved during the hour by the tissue in the right-hand flask. The alkali is then introduced into both flasks, and after allowing a further period for complete absorption of the CO_2 in both flasks the manometer is again read. This reading, which may be called h_2, gives the oxygen absorbed during the hour. Thus the oxygen absorption in mm.^{3} per hour is given by $h_2.k_{O_2}$, and the respiratory CO_2 in mm.^{3} per hour is $(h_1 - h_2).k_{CO_2}$, where k_{O_2} and k_{CO_2} are the constants of the apparatus for oxygen and CO_2 respectively.

Description of the apparatus.

The manometer used is of the usual differential type as described by Dixon and Elliott [1930], but provided with special flasks. The manometer tube contains Clerici fluid instead of the more usual paraffin, which is not heavy enough to deal with the rather large pressure changes involved. This fluid, introduced for this purpose by Dickens and Šimer, consists of a mixture of thallium malonate and thallium formate and has a density of about 4. Before using, it is advisable, after dissolving a small amount of bile salts in it as recommended by Dickens and Šimer, to keep it for a day or two at a few degrees below the room temperature and filter. If this precaution is omitted, the fluid is sometimes liable to deposit crystals in the manometer tube, resulting in a change in density and a blocking of the tube. After this treatment however it will remain unchanged indefinitely, since it is not in contact with rubber as in the Dickens method.

The use of the heavier liquid does not in practice result in any loss of sensitiveness, as the readings themselves become correspondingly more definite and accurate. For instance, a manometer containing paraffin and giving readings significant to 0·5 mm. will under the same conditions give readings significant to 0·1 mm. when Clerici fluid is substituted.

The flasks (Figs. 1 and 2) are of the shape generally used with the Barcroft differential manometer for measurements of tissue respiration. Those which we have used have a capacity of about 40 cc., but smaller flasks may be used if desired. Each flask is provided, on the side opposite to the manometer, with a bulb (*sb*) with a ground joint as shown, so that the fluid contents of the bulb can readily be run into the flask by giving it a half-turn. The upper portion of the bulb is provided with a stopper (*S*) similar to those used by Dickens and Šimer. This serves as an outlet, so that the gas mixture can be passed through the flask in order to fill it, and it can be opened or closed by rotating the stopper. The flask is also provided with a special tap (*T*), communicating with the absorption tube (*at*), for the purpose of introducing the alkali. The bore of this tap does not pass completely through the barrel, but is closed at one end, forming a cup of about 0·5 cc. capacity. Thus by turning the tap this cup can

Fig. 1*a*.

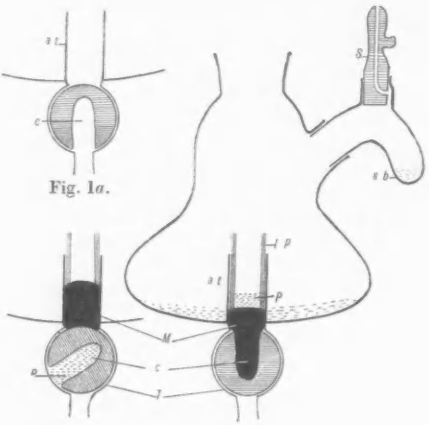

Fig. 1*b*. Fig. 1.

Fig. 1*c*.

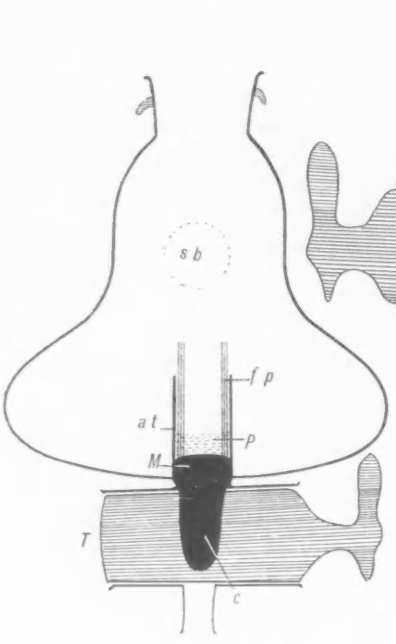

Fig. 2.

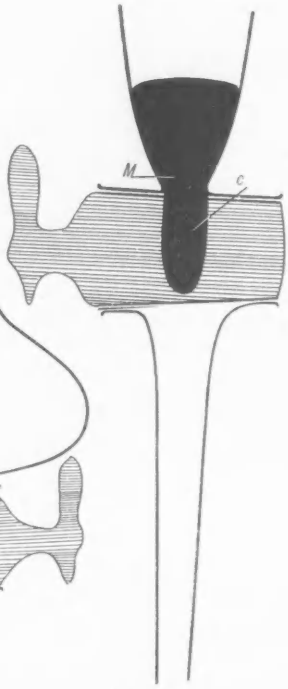

Fig. 2*a*.

be either open to the outside, or open to the absorption tube of the flask or completely closed off. The cup is filled from below with KOH solution and turned so as to close it off; and mercury is placed in the absorption tube, together with a roll of filter-paper (Fig. 1 *b*). If now the tap is turned into the position shown in Fig. 1, the mercury descends into the cup, displacing the alkali upwards into the absorption tube, where it soaks the roll of filter-paper, forming an efficient system for the absorption of CO_2. (See also note on p. 95.)

It should be mentioned that flasks of this type, which may be made of any desired size, can be attached to Barcroft-Warburg manometers and used if preferred in place of the special flasks employed by Dickens and Šimer in their original method. The main advantage of this new type of flask consists in the fact that all the manipulation during the experiment, such as the tipping of the acid into the vessel or the introduction of alkali, can be carried out without tilting the apparatus, or even removing it from the bath. This makes it possible to attach the two flasks to one differential manometer and yet to introduce the acid into either flask independently, which is necessary in the present method.

A number of points relating to the construction of the apparatus may be mentioned here. In the first place it is essential that the two flasks of each instrument should be of equal volume (within 0·1 % if possible). If this is not so, the CO_2 introduced in the form of bicarbonate in the medium will not be properly balanced out, and an error will be introduced into the determination of the respiratory CO_2. This does not in any case affect the values of oxygen uptake, and only affects the CO_2 values appreciably when the amount of bicarbonate in the medium is large in comparison with the CO_2 produced by the respiration. For instance, a 1 % inequality in the flasks will produce an error in the CO_2 equal to 1 % of the bicarbonate added, which may be equivalent to an error of several per cent. in the CO_2 of respiration. We have however experienced no difficulty in obtaining flasks of the desired degree of equality[1], and indeed it is not a difficult matter to adjust the volume with great precision during manufacture by expanding or contracting the side bulbs slightly and by grinding in the joints to a greater or less extent.

All the joints and taps must be very well ground, and capable of closing hermetically with the minimum amount of grease. This applies especially to the stoppers *S*.

The glass at the bottom of the flasks must not be too thin, as this has to carry the tap for the introduction of alkali, and the strain produced when turning this may be appreciable if the tap grease is cold. For this reason it is advisable to hold the outside of the tap while turning it.

It is convenient to have the handles of the bottom taps orientated towards the outer sides of the flasks (the right- and left-hand flasks thus being mirror images of one another), which makes them accessible for turning when the vessels are immersed in the bath.

The tube of the manometer should have a uniform bore, and the cross-section of the bore should be between 0·6 and 1·5 mm.[2] We find that with tubes smaller than this the Clerici liquid does not run quite freely, while with large tubes additional terms must be introduced into the formula for the constants given below.

For convenience of handling the flasks during filling a wooden stand (Fig. 3) is used, having openings cut out as shown so as to allow the flasks

[1] The instruments used by us were made by Messrs W. G. Flaig and Sons, Hatton Garden, London.

to be supported with the bottom taps turned in any position. The same stand can be used for draining the vessels after washing.

The grease used is anhydrous lanoline ("Adeps Lanae"). This is very satisfactory for all joints except the bottom taps, and does not become too liquid at the higher temperatures used. For the bottom taps we find a somewhat stiffer rubber grease advisable.

All the joints and taps are secured by rubber bands stretched fairly tightly.

The constants of the apparatus for oxygen and CO_2 are obtained by measuring the volumes of the flasks and the cross-section of the manometer tube by filling with mercury and weighing in the usual way; and then by calculation from the following formula, modified from the formula of Warburg [1926] by

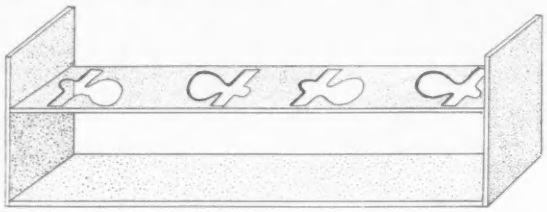

Fig. 3.

the omission of terms which become negligible when Clerici fluid is used and allowing for the fact that the manometer of the Barcroft apparatus is usually made with a tilt in order to facilitate reading:

$$k = \left(\cos\theta + \frac{AP_0}{2v_G}\right)\left(\frac{v_G\frac{273}{T} + v_F\alpha}{P_0} + \frac{A}{2}\frac{273}{T}\right),$$

where θ = angle of manometer with the vertical,

A = cross-section of manometer tube in mm.^{2},

P_0 = normal pressure (760 mm. Hg) in mm. Clerici fluid,

v_G = volume of gas space in each flask in mm.^{3},

T = absolute temperature of experiment,

v_F = total volume of liquid in each flask in mm.^{3},

α = solubility of the gas in question (O_2 or CO_2) in the liquid.

In this formula θ and A are measured, T and v_F are known from the conditions of the experiment; v_G is obtained by subtracting from the volume of the flask (which must of course include that of the manometer tube to which it is connected) the volume of the mercury added to the absorption tube and of the liquid (medium and acid) added to the flask. α for various values of T can be found in tables of physical constants. The density D of the Clerici fluid must be carefully determined, and P_0 is then given by $P_0 = 760\frac{13{\cdot}60}{D}$. By multiplying the reading of the manometer in mm. by this constant the result is obtained directly in mm.^{3} of dry gas at N.T.P., so that no further correction is required. The constants for oxygen obtained by this method have been checked experimentally by an improved method on the principle of Münzer and Neumann [1917] and the results were found to agree to within 1 %. The constants for CO_2 are not readily obtained by the latter method, but they have been checked by the chemical method and found to agree equally well.

For filling the flasks with the gas mixture the arrangement shown in Fig. 4 is used. The gas is led from the cylinder through glass or metal tubing (rubber tubing is avoided as far as possible to avoid loss of CO_2 from the gas mixture by diffusion). At *E* is a branch tube dipping into a cylinder of water to act as an escape for the gas in case the pressure accidentally becomes too great. The gas passes first through a flowmeter *F* and then through a bulb *B* which contains water and is immersed in the thermostat. This is used, as in the technique of Dickens and Šimer, for the purpose of saturating the gas with water vapour before it enters the apparatus, so as to prevent loss of water from the flasks by evaporation. The gas is then distributed by the branched tubes shown, which are fixed at the back of the thermostat and provided with lengths of rubber pressure-tubing for connection to the manometers, so as to allow the latter to be shaken in the thermostat while the gas is passing.

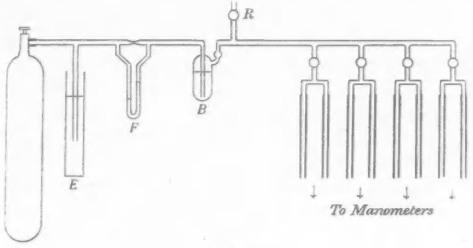

Fig. 4.

Experimental procedure.

The manipulations of filling are carried out in the following order.

(1) The flasks are inverted, the bottom taps greased, inserted into position and turned so that the cups open to the outside (Fig. 1 *a*). By means of a fine pipette the cups are completely filled with a 40 % solution of KOH, and the taps turned as in Fig. 1 *b* so as to enclose the alkali without including any air bubbles. The excess of KOH solution is removed from the outer tubes.

(2) The flasks are placed upright in the stand, and a definite amount of mercury (about 0·7 cc.) is accurately delivered directly into each absorption tube from a special burette (Fig. 2 *a*) constructed on the same principle as the bottom taps of the flasks. Care must be taken to see that no droplets fall into the flasks outside the tubes. Rolls of No. 40 Whatman filter-paper are then inserted into the tubes. They should be opened out so that they fit the tubes fairly tightly, and should be pushed in so as to reach the bottom of the tubes. The paper should project about 5 mm. above the tops of the tubes.

(3) The side bulbs are placed in position, 0·3 cc. of 3*N* HCl is measured into each, and the stoppers are inserted as shown in the figure. A small glass tube as shown in Fig. 1 *c* is then loosely attached to the upper end of each stopper by means of a short piece of rubber tubing. These tubes are of such a length that when the apparatus is placed in position in the water-bath their open ends dip below the surface of the water.

(4) The medium (3·0 cc.), previously brought into equilibrium with the gas mixture, is introduced, together with the tissue, into the flasks. As it is important that exactly equal amounts of the bicarbonate-containing medium should be added to the two flasks, this is best done from a micro-burette.

(5) The flasks are attached to the manometer, secured with rubber bands, and the apparatus placed in position in the thermostat. The rubber tubes supplying the appropriate gas mixture are attached to the free ends of the manometer tubes, and the gas is passed through both flasks simultaneously, escaping by way of the stoppers *S* and the small tubes attached to them through the water of the bath. The manometer taps should be so adjusted that the gas passes through the two flasks at approximately the same rate. The use of the small bubbling tubes attached to the outlets makes it easy to see when this is the case. The stream of gas is adjusted by means of the flowmeter so that about 200 cc. per min. passes through each flask, and this is continued for at least 10 mins., during which time the apparatus is shaken, as it is essential that both flasks should be completely filled with the mixture. While the gas is passing, and without removing the apparatus from the thermostat, all the ground joints are carefully worked in so that they bind moderately tightly. This is absolutely essential for the success of the method, as also of the original Dickens and Šimer method, owing to the considerable pressure differences involved. The bottom taps are also worked in somewhat, though it is not necessary to carry this as far as with the other joints. At the end of 10 mins. the tap *R* (Fig. 4) is opened, the stoppers *S* are closed and worked in, the bubbling tubes removed, and the apparatus is shaken for a further 5 mins. with the rubber supply tubes still attached, in order to allow the temperature of the gas to rise completely to that of the water-bath; after which the taps of the manometer are closed, the rubber tubes detached, and the experiment carried out as described above.

After each experiment the flasks are emptied, rinsed, all the parts separated, wiped, and cleaned thoroughly from grease in concentrated sulphuric-dichromate cleaning mixture, and finally washed in tap and distilled water and drained.

To illustrate the working of the method we may quote an experiment carried out with bakers' yeast, in which parallel experiments were made with the same yeast by the original method of Dickens and Šimer and by the direct method in the absence of bicarbonate in the ordinary Barcroft apparatus. For this experiment a suspension of yeast (4 mg. moist weight per cc.) was made up in Ringer solution containing 0·025 *M* bicarbonate and 1 % of glucose, previously saturated with a mixture of 5 % CO_2 and 95 % O_2. This suspension was used for the determinations by the new method and the method of Dickens and Šimer. Another suspension, containing the same amounts of yeast and glucose in phosphate buffer of the same p_H, was used for the direct method. The temperature of the experiment was 38°. 1·5 cc. of the first suspension (6 mg. of yeast) were used in the Dickens-Šimer manometers, 3·0 cc. of this suspension (12 mg. of yeast) were used in the new apparatus, and 3·0 cc. of the second suspension (12 mg. of yeast) in the simple Barcroft apparatus. The Dickens-Šimer manometers and the new apparatus were filled with the $CO_2 + O_2$ mixture, and the Barcroft apparatus with oxygen. In the Barcroft apparatus the CO_2 produced by the yeast was absorbed by "KOH-papers" in the usual way, as described by Dixon and Elliott.

For purposes of illustration readings of the new apparatus were recorded every 5 mins., and are plotted in the curve shown in Fig. 5. In actual practice of course two readings only are necessary. At the point *A* on the curve the acid was run into the left-hand flask, at the point *B* the acid was run into the right-hand flask, and at *C* the alkali was introduced into both flasks. The reading h_1 was + 0·1 mm., and the final reading h_2 was − 34·1 mm. The constant of the apparatus for oxygen was 13·88, and that for CO_2 was 14·58. Thus

the oxygen usage during 45 mins. was $-34{\cdot}1 \times 13{\cdot}88 = -473$ mm.^{3}, while the CO_2 output during the same period was

$$[+0{\cdot}1 - (-34{\cdot}1)] \times 14{\cdot}58 = +498 \text{ mm.}^3$$

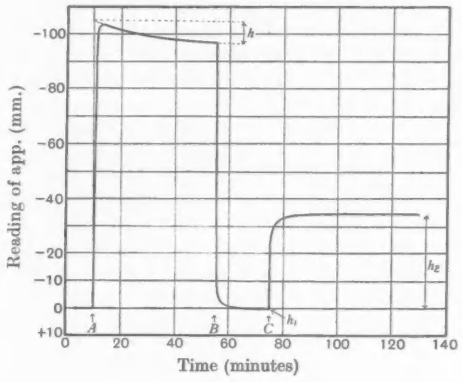

Fig. 5.

The results given by the three methods are shown in the following comparative table.

Method	Dickens and Šimer	New method	Direct Barcroft
Medium	Bicarbonate Ringer + CO_2		Phosphate
Amount of yeast...	6 mg.	12 mg.	12 mg.
O_2 in 45 mins.	−232	−473	−471
CO_2 in 45 mins.	+252	+498	—
R.Q.	1·08	1·05	—
O_2 per mg. per hour	− 51·6	− 52·5	−52·4
Acid production	+ 50	+108	—

In working out the results due regard must be paid to the sign of the readings. We have followed the usual convention, according to which an amount of gas evolved by the tissue is considered as positive and an amount of gas absorbed negative. Accordingly readings which indicate an absorption of gas in the right-hand flask (in which the respiration takes place), or an evolution in the left-hand flask, are taken as negative, and *vice versa*.

It should be pointed out also that the significant readings in this method are the actual readings of the manometer, whereas in the method of Dickens and Šimer it is always the change in reading which is used in the calculations.

The readings during the half-hour following the introduction of the alkali have no significance; in fact, the manometric fluid may move in either direction at first. During this time a considerable amount of CO_2 is being absorbed in both flasks simultaneously, and the direction of movement of the manometer is simply determined by the relative rates of absorption in the two flasks, which is in turn determined by such factors as the area of filter-paper exposed, *etc.* As the absorption approaches completion however the reading comes quite definitely to its final value, irrespectively of the earlier movements. It must be emphasised that mere constancy in manometric reading does not necessarily indicate that the absorption of CO_2 is complete: it may only show that the

absorption is proceeding at the same rate in both flasks. It is essential to wait for at least 45 mins. after the introduction of the alkali before taking the final reading. Neglect to do this may give rise to serious error.

Dixon and Elliott found that filter-paper soaked with strong KOH solution absorbed oxygen. With 40 % KOH and No. 40 (starch-free) filter-paper this uptake was however found to be small, even at the higher temperatures, and we found that provided that roughly equal amounts of filter-paper were taken for each flask this effect was completely compensated and could safely be neglected.

In the case of animal tissues, when bicarbonate-Ringer solution is used as the medium, a single experiment by the new method will give not only the respiratory CO_2 and oxygen but also if desired the aerobic glycolysis as measured by acid production. It is only necessary to determine the change in the manometric reading during the respiration period (which may be called h), and the aerobic glycolysis is then given by $(h - h_1).k_{CO_2}$. This will be clear from the consideration that the change of reading h represents the algebraical sum of the oxygen and CO_2 of respiration and the change in the amount of bound CO_2 (*i.e.* bicarbonate), while h_1 represents the sum of the oxygen and CO_2 only. In bicarbonate-Ringer solution, where "retention" of CO_2 does not occur, the change in the bicarbonate is equal to the aerobic glycolysis. In serum or phosphate buffer however some of the respiratory CO_2 is retained as bicarbonate, and the change in bicarbonate is therefore not due to the glycolysis alone. In serum a more complicated procedure would be necessary to determine the glycolysis, as in the method of Dickens and Šimer. Of course neither retention of CO_2 nor glycolysis affects the values of the respiratory exchanges determined by the method, as excess of acid is added before the determination of h_1 and h_2.

h is the change in the manometric reading produced by the activity of the tissue during the period of the experiment. As however the evolution of the bound CO_2 on addition of the acid to the left-hand flask is not instantaneous, the movement of the manometer during the first few minutes of the period is due to this cause as well as to the activity of the tissue. In order to obtain h it is therefore necessary to determine the rate of change during the period after the evolution of CO_2 in the left-hand flask is complete, and to extrapolate back to the beginning of the period. As the evolution of CO_2 is rapid, and reaches completion in a few minutes, this extrapolation is a simple matter. The method of determining h will be clear from Fig. 5.

In the experiment quoted h was found to be $+ 7{\cdot}5$ mm. As h_1 was $+ 0{\cdot}1$ the acid production was $(+ 7{\cdot}5 - 0{\cdot}1) \times 14{\cdot}58 = + 108$ mm.^{3} This means that during the 45-min. period an amount of acid equivalent to 108 mm.^{3} of CO_2 had been produced by the 12 mg. of yeast. In the duplicate experiment by the original Dickens and Šimer method the acid production was found to be $+ 50$ for 6 mg. of yeast.

The theory and principle of the method can be summarised as follows. During the experimental period oxygen is being absorbed, CO_2 produced and glycolysis (acid production) taking place. The reading h produced is the resultant of these three processes. After the acid has been introduced the whole of the bicarbonate is converted into free CO_2, and the effect of the glycolysis is thereby eliminated. As equal amounts of bicarbonate were initially present in the two flasks this CO_2 balances out, and the reading h_1 is thus the resultant of the oxygen uptake and the respiratory CO_2 production. After the alkali has been introduced the whole of the CO_2 is absorbed, and since the same amount of CO_2 was initially present in both flasks the resulting reading is determined

by the oxygen uptake alone. Thus, if h_{O_2}, h_{CO_2} and h_G are the readings which would be produced by the oxygen uptake, the respiratory CO_2 output and the glycolysis respectively if these processes took place separately,

$$h = h_{O_2} + h_{CO_2} + h_G,$$
$$h_1 = h_{O_2} + h_{CO_2},$$
$$h_2 = h_{O_2}.$$

The method can be adapted to a number of purposes other than the measurement of the respiration of tissues. It is especially useful for studying the oxidation of substances added to the tissue or tissue preparation. In this case the tissue is added as before to both flasks, but the substance whose oxidation it is desired to investigate is added to the right-hand flask only. It is best to place it in a small cup provided with a hook by which it can be suspended from the edge of the absorption tube (as used by Keilin [1929]). This can readily be dislodged so as to fall into the medium after the equilibration at the beginning of the experiment. The acid is not run into either flask until the end of the experimental period, when it is run into both flasks simultaneously. The remainder of the procedure is the same as before. By this means one obtains directly the oxygen uptake and the CO_2 output due to the added substance, already corrected for the spontaneous respiration of the tissue itself.

SUMMARY.

A modification of the second method of Dickens and Šimer [1931] is described, by which the oxygen and CO_2 of respiration of a tissue or cell suspension may be determined, using as medium either phosphate buffer, or bicarbonate-Ringer solution or serum in equilibrium with the physiological tension of CO_2. In this method only a single manometer is used, and only two readings and a minimum of calculation are required. In the case of bicarbonate-Ringer solution a third reading gives also the aerobic glycolysis.

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Note added February 10, 1933. If it is desired to avoid the presence of mercury in the flasks, glass rods may be used instead for displacing the alkali. A number of equal lengths (2–3 cm.) are cut from a glass rod of such a diameter that it will go easily into the cups of the absorption taps. They are ground down if necessary until they are of all the same weight, and the ends rounded in the flame. One of these is placed in each absorption tube inside the roll of filter-paper and drops into the cup when the tap is turned. The rods should all be of the same volume so that they may be interchanged without altering the constants.

XIII. A VACUUM EVAPORATING PLANT FOR LABORATORY USE.

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(*Received December 10th, 1932.*)

In the course of investigations on the metabolic products of moulds it has frequently been necessary to concentrate, at low temperatures, volumes of liquid of 20 to 60 litres. The apparatus here described was designed to meet our needs and was constructed for us by Messrs Brown and Son (Alembic Works), Ltd., Wedmore Street, Holloway, London, N. 19. It is thought that a description will be of interest to others who are faced with the same problem of concentrating comparatively large volumes of aqueous solutions for the isolation of substances of low thermal stability. A photograph of the plant is given in Fig. 1, and a scale drawing in Fig. 2.

The evaporation is carried out in six 5-litre bolt-head flasks (*A*), immersed to the necks in a water-bath (*B*) maintained usually at 50°. The temperature of the liquid in the flasks, with evaporation in progress, is 20–25°. In an earlier apparatus, of somewhat similar design, the receiver was cooled by means of chilled brine, but much trouble was experienced by choking of the delivery tubes and by a gradual falling off in efficiency due to the deposition of ice on the surface of the cooling coils. In the present plant, therefore, tap-water is relied on for condensation of the distillate, a somewhat lower theoretical efficiency, due to a less steep temperature gradient, being offset by convenience of operation and absence of periodical breakdown. Vacuum is maintained in the apparatus by means of a Model A 1 or A 2 rotary oil-pump, supplied by Messrs W. Edwards and Co., Allendale Road, Denmark Hill, London, S.E. 5, and fitted with a special water-discharge trap, which is substantially the same as that described by Askew and Bourdillon [1932]. The pressure during distillation is usually slightly less than the vapour pressure of water at the temperature of the cooling water.

The plant is constructed mainly of copper, heavily tinned on all surfaces exposed to water or water-vapour. It is 5′ 5″ in total height and 4′ 10″ in overall diameter. The water-bath (*B*), in the form of a circular trough surrounding the condenser (*C*), is 12″ wide and 12″ deep. It is fitted with a draw-off cock (*D*) going to drain, and with two metal pockets for holding thermometers (not shown). It is supported on a strong iron stand (*E*), the top of the bath being 4 ft. from the floor. Surrounding the bath, at a height of 30″, is a shelf (*F*), 11″ wide, of cypress wood, which accommodates six glass jars (*G*), of 6 litres capacity, containing the liquid to be evaporated.

The bolt-head flasks, spaced at equal distances around the bath, are held by clamps (*H*) made of flat iron bar, lined with cork. The clamps stretch across the top of the water-bath, are held in short, slotted metal posts riveted to the sides of the bath and secured by simple catches on the turn-button principle. The flasks are fitted with rubber bungs suitably bored to take the glass feed-tubes (*I*) and the metal vapour-tubes (*J*). The latter are made of brass, tinned inside and out and are 1″ in diameter. They are fastened to the header of the condenser by

well made brass unions (*K*), the joints being made with thin leather washers soaked in castor oil. Such joints are easily made and broken without movement of the parts to be joined.

The condenser, 2 ft. long exclusive of head, and 8″ in diameter is supported on an iron ring attached to the main frame, and is insulated from the water-bath surrounding it by a packing of slag-wool (*L*). The delivery end of the condenser is attached by means of a brass union (*M*) to a cylindrical receiver (*N*) of 40 litres

Fig. 1.

capacity, fitted with an internal copper coil (*O*) through which the cooling water flows before supplying the condenser proper. A tap (*P*) between the coil and the condenser prevents back flow of water should it be necessary to detach the receiver. Levelling screws (not shown) are provided for securing accurate alignment of receiver and condenser so that no strain is put on the union connecting them. The receiver is fitted with a nozzle (*Q*) for connecting to the vacuum pump, a draw-off tap (*R*) at the bottom, an air valve (*S*) in the top for breaking vacuum, and a gauge-glass (*T*) for showing the level of the condensate.

The feed of liquid to the flasks is through glass tubes bent twice at right-angles. One limb of the feed tube (*I*) is constricted at the end and passes through

the rubber bung almost to the bottom of the flask. The other limb is provided with a leaky glass cock (*U*) and ends in a short length of rubber tubing which dips into the feed liquid contained in the glass jar (*G*). The tap is set so that a slow feed of liquid mixed with air bubbles is carried into the flask at approximately the rate to balance evaporation.

The water-bath is heated by a large ring gas-burner (*V*) (supporting struts, from main frame, not shown), the gas being fed in at two diametrically opposite points in order to secure approximately even heating all round the bath. When

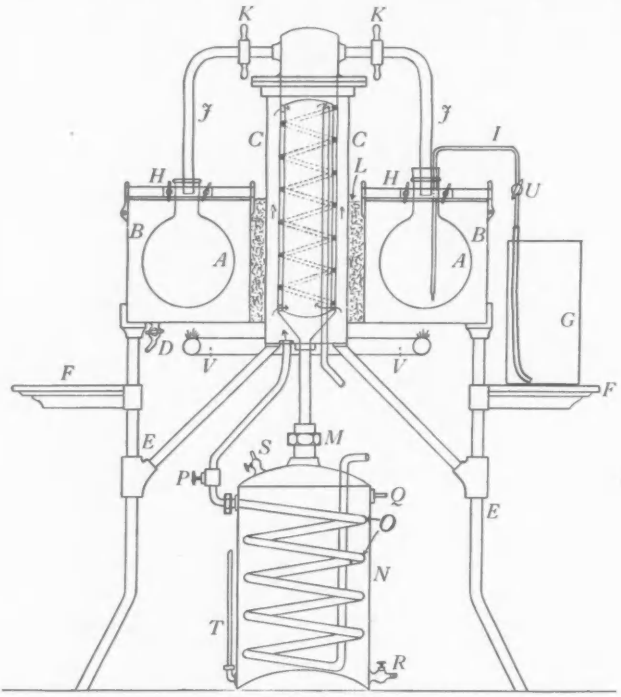

Fig. 2. (Scale = 1/16.)

starting up the plant it is found convenient to fill the bath with water at approximately the desired temperature. The gas flame is then readily adjusted to maintain the temperature with an accuracy of $\pm 1°$.

The plant has been in use for more than a year and has proved very satisfactory. Liquids which do not froth unduly may be evaporated at the rate of $3\frac{1}{2}$ to 4 litres per hour. With troublesome liquids it has sometimes been necessary to run the evaporation with not more than about 150 cc. of liquid in each flask. With the comparatively small liquid surface exposed, the rate, under these conditions, is usually $2\frac{1}{2}$ to 3 litres per hour, or about 25 litres per day.

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XIV. THE DENATURATION OF PROTEINS.

X. THE ACID AND ALKALI TITRATION CURVES OF CRYSTALLINE EGG-ALBUMIN.

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(*Received December 12th, 1932.*)

The subject of the titration curves of proteins is one to which a great deal of attention has been paid, both in this laboratory and elsewhere, and although substantial concordance is to be found in the results of various workers using different methods, there are nevertheless certain discrepancies. Thus Booth [1930] working with crystalline egg-albumin, obtained a titration curve which shows a sharp inflection below p_H 3·0. This inflection does not appear in the titration curves obtained by other workers, *viz.* Loeb [1924], Hendrix and Wilson [1928], Wu, Liu and Chou [1931], and more recently by Prideaux and Woods [1932].

Prideaux and Woods for this reason, and for others which they mention, ignore Booth's curves in their comparison of the available titration data, but as will be shown in the subsequent discussion, these reasons are based on a misunderstanding of the purpose of Booth's work. However, that there should be conflicting experimental evidence is most unsatisfactory, and it was therefore considered worth while to obtain a titration curve using the improved glass electrode method for measuring the p_H values, to determine whether or not the inflection does exist, and, from all the available data to consider whether the conclusion of Booth, *viz.* that on denaturation there is no change in the acid or alkali titratability, is true.

Experimental.

The crystalline egg-albumin was prepared by the method of Sørensen [1917], the stock solution being freed from salt by prolonged dialysis. The concentration of protein in the stock solution was estimated by the method of Devoto as modified by Hopkins [1900].

The method of titration employed was such as to give the same final volume of protein solution, and also the same final protein concentration in each case. With this object, the volume of stock albumin solution which when diluted to 20 cc. would give a 0·3 % solution was measured out. In the first case the dilution was performed with distilled water, and after mixing well the p_H of the resulting solution was measured using the improved glass electrode technique of Harrison [1930], the temperature used being 18°. In the next solution the dilution was performed with 1 cc. less of distilled water, *plus* 1 cc. of a dilute standard sulphuric acid solution, and so on. An exactly similar series of experiments was then performed using a standard alkali solution instead of acid.

To obtain the blank corrections which must be applied to allow for the volume of acid or alkali necessary to adjust the p_H of the medium in which the albumin is dissolved, it is necessary to know at each p_H the volume of standard acid which is present in 20 cc. of acid-water solution. These corrections can be obtained from the table given by Harris [1923] in the form cc. of 0·1 *N* acid which are necessary in 100 cc. acid solution to give the required p_H. Thus if the titration results obtained are recast in the form cc. of 0·1 *N* acid present in 100 cc. of 0·3 % egg-albumin solution, a simple subtraction gives the volume of 0·1 *N* acid combined with the albumin in the solution, and from this the volume combined with 1 g. of albumin is easily obtained, thus giving the results in a form which can readily be compared with those of the other workers. The titration tables given below show the results in this form in the last column.

Table I. *Titration of crystalline egg-albumin with acid at 18°.*

p_H	cc. 0·0834 *N* acid per 20 cc. 0·3 % albumin solution	cc. 0·1 *N* acid per 100 cc. 0·3 % albumin solution (*a*)	Blank correction (Harris) (*b*)	Corrected titration per 1 g. albumin $\frac{(a-b)\times 10}{3}$
5·08	0·00	0·00	0·00	0·00
4·26	0·12	0·50	0·00	1·67
3·69	0·25	1·04	0·00	3·47
2·76	1·00	4·17	2·00	7·23
2·31	2·00	8·34	5·60	9·13

Table II. *Titration of crystalline egg-albumin with alkali at 18°.*

p_H	cc. 0·0978 *N* NaOH per 20 cc. 0·3 % albumin solution	cc. 0·1 *N* alkali per 100 cc. 0·3 % albumin solution	Blank correction (Harris)	Corrected titration per 1 g. albumin
5·08	0·00	0·00	0·00	0·00
5·89	0·05	0·24	0·00	0·80
7·02	0·10	0·49	0·00	1·63
9·74	0·30	1·47	0·00	4·90
10·12	1·00	4·89	0·80	13·63

To facilitate comparison of these results with those of the other investigators, they must be recast so as to represent titrations from the isoelectric point p_H 4·7. This can be done quite simply by plotting the results as a curve, when it is found that p_H 4·7 represents the addition of 0·7 cc. of 0·1 *N* acid per 1 g. of albumin to a solution at p_H 5·08. Thus to represent titrations from p_H 4·7 all the acid titrations given above must be diminished by 0·7 cc. and all the alkali titrations increased by the same amount. The results obtained are given in Table III in comparison with a typical series of results of Prideaux and Woods [1932] obtained from titration experiments on 1 % solutions using the hydrogen electrode, a series of Loeb [1924] using 1 % solutions and an electrometric method of measurement, and finally with Booth's results using the quinhydrone electrode (these latter recalculated to represent titrations as cc. 0·1 *N* acid per 1 g. albumin after the requisite correction has been made, and measured from p_H 4·7). It should be noted that all the results given should be quite comparable since in the form presented they are independent of the concentration of the solutions in which the titrations were actually performed. The only real difference is that the writer's data hold for a temperature of 18° instead of 25°. This slight difference will not in any way affect the conclusions to be drawn later.

In Table III all the titrations (T) are measured from the isoelectric point and are given in cc. of 0·1 *N* acid per g. of albumin, the negative values being, of course, obtained for titrations with alkali.

Table III.

Loughlin		Prideaux and Woods			Loeb		Booth	
T	p_H	T	p_H		T	p_H	T	p_H
−5·60	9·74	−6·00	—	9·85	—	—	—	—
—	—	−5·00	—	9·45	−5·0	9·50	—	—
—	—	−4·00	9·05	8·65	−4·0	8·75	—	—
−2·33	7·02	−3·00	7·00	6·90	−3·0	7·50	—	—
−1·50	5·89	−2·00	6·00	—	−2·0	6·00	−1·57	6·94
−0·70	5·08	−1·00	5·30	—	−1·0	5·25	−0·74	5·68
0·00	4·70	0·00	4·70	—	0·0	4·80	0·00	4·78
—	—	0·22	4·60	—	0·3	4·60	—	—
—	—	0·65	4·40	—	0·7	4·40	0·76	4·12
0·97	4·26	1·09	4·25	—	1·1	4·20	—	—
—	—	1·74	4·05	—	1·7	4·00	—	—
—	—	2·20	3·85	—	2·2	3·80	2·12	3·51
2·77	3·69	2·96	3·65	—	2·9	3·60	—	—
—	—	3·84	3·35	—	3·5	3·40	3·56	3·08
—	—	4·60	3·20	—	4·8	3·20	4·19	2·72
—	—	6·00	2·90	—	5·3	2·80	4·22	2·33
6·53	2·76	7·10	2·70	—	6·5	2·60	—	—
8·43	2·31	—	—	—	—	—	—	—
—	—	9·63	2·50	—	—	—	—	—

The figures in the above table are plotted on the same axes in Fig. 1.

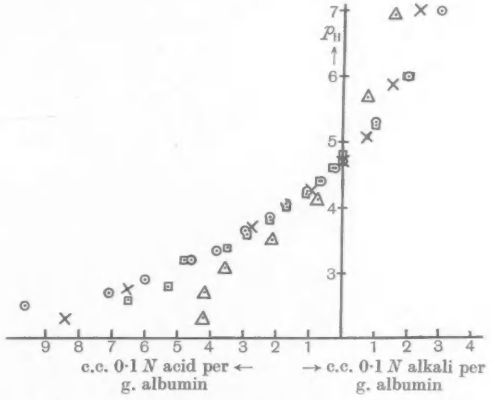

Fig. 1. Corrected titration curves of undenatured egg-albumin.

× × Loughlin.
⊙ ⊙ Prideaux and Woods.
⊡ ⊡ Loeb.
△ △ Booth.

From this diagram several points are at once obvious. In the first place, although Booth's curve is of the same type as the other three curves given, it is the only curve which shows any marked inflection in the acid region. It is only right to infer therefore, that this inflection, depending as it does almost entirely on one

reading, is due to experimental error, and that the true titration curve continues practically linearly towards the very acid regions as shown in the other curves.

The second point, which is one of some importance, is that the writer's curve, obtained by the use of the glass electrode, agrees very well with the curves of Prideaux and Woods using the hydrogen electrode, over a range from p_H 6·0 down to p_H 2·5 showing that the glass electrode gives a very convenient and accurate method of measuring the p_H values of protein solutions over this range.

At p_H values on the alkaline side of p_H 6·0 very poor agreement is obtained between the titration curves of different workers, and even between different curves given by the same investigators on different solutions. Taking the curves of Prideaux and Woods for example, two different types of curve are obtained, one which rises fairly rapidly to p_H 10 on the addition of only 6 cc. 0·1 *N* NaOH per g. of albumin, and a second which scarcely rises above p_H 9 even after the addition of 12 cc. 0·1 *N* NaOH. For this reason the titration curves will be considered only on the acid side of p_H 6·0.

Discussion.

It has been shown above that Booth's titration curve does not agree very well either with the curves of the writer, or with the curves of other investigators, and furthermore the inflection reported by Booth is peculiar to his curve. The question now arises whether these facts cast doubt on the validity of Booth's conclusion that on denaturation there is no alteration in the acid or alkali titratability of crystalline egg-albumin. The best method of answering this question is by making a critical examination of the available titration data for both undenatured and denatured albumin.

The curve for undenatured egg-albumin has been obtained by Prideaux and Woods [1932], by Loeb [1924] and by Wu, Liu and Chou [1931] using the hydrogen electrode; by Hendrix and Wilson [1928], and Wu, Liu and Chou using the quinhydrone electrode, and by the writer using the glass electrode, and it is found that there is excellent agreement between all six from p_H 6·0 down to fairly acid regions (about p_H 2·5). Consequently the curve for undenatured egg-albumin may be regarded as well-defined.

For denatured albumin the following curves have been obtained—albumin denatured by alcohol and good commercial dried albumin (probably denatured) by Prideaux and Woods using the hydrogen electrode; albumin denatured by heat by Hendrix and Wilson using the quinhydrone electrode, by Wu, Liu and Chou using both the quinhydrone and hydrogen electrodes, and by Booth using quinhydrone.

Prideaux and Woods, referring to all their curves both for undenatured and denatured albumin, state that "the results of all series appear to be in satisfactory agreement up to and slightly beyond p_H 5·0. Any given addition of acid defines the p_H within about $\pm$ 0·2."

Wu, Liu and Chou on the other hand state that the titration curve of the albumin is shifted to the alkaline side by denaturation. They then continue "It will be noted also that the greater portion of the titration curves of the natural and the denatured albumin are nearly parallel. If the titration curve of the denatured albumin is shifted toward the acid side by about 0·3 of a p_H unit, it will practically coincide with that of the natural albumin." On examining the data given, however, we find that the two sets of titrations as given are not strictly comparable, for whereas the undenatured solutions are titrated from p_H 7·00 the denatured solutions are titrated from p_H 7·25. This was done because during the

heating, the p_H of the albumin solution used increased on denaturation from 6·96 to 7·25. Thus the denatured titrations given are really the amounts of acid which would have to be added to an undenatured albumin solution at p_H 7·0 to bring it to the required p_H after denaturation. To render the titration curve of these workers comparable with the others mentioned above it is necessary to recalculate the data given to represent titrations from p_H 4·7–4·8. When this is done the undenatured and denatured curves represent titrations from a common origin and the 0·25 difference in p_H is removed. In other words, the titration curve of the denatured albumin is shifted toward the acid side by about 0·3 of a p_H unit and thus does in fact coincide with that of the native albumin. The writer has recalculated the data of Wu, Liu and Chou as titrations from p_H 4·7, and finds that not only do the curves coincide with each other but also with the single curve based on the data for undenatured albumin obtained by Prideaux and Woods, Loeb, and by himself (Fig. 2). Thus these results may be taken as confirming rather than disproving the identity of the titratability of undenatured and denatured egg-albumin. This point is emphasised by Fig. 2.

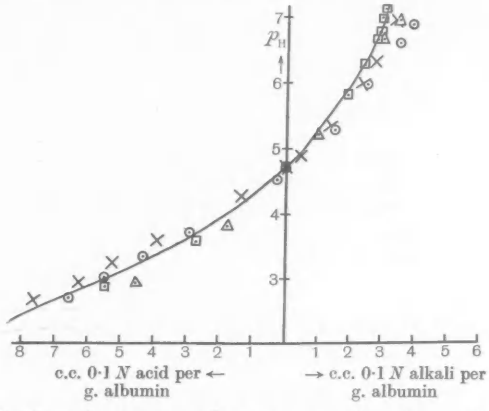

Fig. 2. Comparison of corrected titration curves for undenatured and denatured egg-albumin.

Undenatured albumin: ——— Composite of results of Loughlin, Loeb, Prideaux and Woods.
Undenatured albumin: × × Wu, Liu and Chou using quinhydrone electrode.
Undenatured albumin: ⊡ ⊡ Wu, Liu and Chou using hydrogen electrode.
Denatured albumin: ⊙ ⊙ Wu, Liu and Chou using quinhydrone electrode.
Denatured albumin: △ △ Wu, Liu and Chou using hydrogen electrode.

Finally, considering the curves of Hendrix and Wilson, it is found that here a definite alteration on coagulation is reported. The curves given show that the uncoagulated albumin combines with more HCl or NaOH than the coagulated albumin at any given p_H. Two curves are given for coagulated albumin, the first using albumin which had been coagulated by heating for about 30 minutes in an autoclave at atmospheric pressure, after which it was allowed to stand overnight and then filtered. The precipitated albumin was well washed with hot water and subsequently dried as much as possible by pressing between filter-papers and washing with absolute alcohol. After it was thoroughly dried in an oven at 60° it was pulverised and weighed for titration. The second curve is for albumin heated as above but titrated in the mother-liquor without being filtered off. It is

significant that this second curve, *i.e.* the one for coagulated unfiltered albumin, corresponds more closely with the curve for uncoagulated albumin than does the one for dried pulverised coagulated albumin. In fact, in view of the agreement obtained above by other workers for the curves of undenatured and denatured albumin, it seems probable that the alteration observed here is due to the unnecessarily drastic methods employed for the coagulation.

It seems justifiable, therefore, to conclude from the mass of data available that, provided the albumin is denatured by not too drastic methods, the curves for the undenatured and the denatured protein are congruent over a range of p_H from 6·0 to 2·5.

Above p_H 6·0, as all the investigators mentioned have found, wide variations of p_H are obtained for a given addition of alkali even between different experiments in the same series. It is therefore not possible to draw a similar conclusion above p_H 6·0. There are still, however, the alkali formaldehyde titration curves of Booth [1930], which have shown that under identical conditions the undenatured and denatured albumin are indistinguishable in their behaviour. It should be noted that Booth was not trying to obtain an alkali titration curve for egg-albumin itself. His object was to determine whether the undenatured and denatured protein acted similarly or otherwise under identical conditions, on the addition of alkali. His curve is therefore not intended to be compared with the ordinary titration curves when only albumin and alkali are present.

Booth used in his experiments the formaldehyde titration method of Sørensen [1907] for the titration of amino-acids. The addition of formaldehyde has the effect of blocking the amino-groups and so enhancing the acidic nature of the protein. The fact that the titrations obtained are identical for undenatured and denatured albumin cannot therefore be ignored as meaningless but rather indicates that the conclusion drawn above at p_H values more acid than 6·0 should also hold on the alkaline side of p_H 6·0.

One may conclude therefore that the acid and alkali titratability of egg-albumin is the same for the undenatured and denatured protein at p_H values on the acid side of p_H 6·0, and probably also on the alkaline side, although up to the present the latter point has not been demonstrated in an entirely satisfactory way.

Summary.

1. A titration curve for undenatured crystalline egg-albumin has been obtained by the use of the improved glass electrode method.

2. It is shown that this curve is in excellent agreement with the curves of other workers obtained by the use of the hydrogen or quinhydrone electrodes.

3. No inflection in the curve is detected in the acid region.

4. Titration curves obtained by many investigators for both undenatured and denatured egg-albumin using different methods are recast in a form suitable for comparison, and when this comparison is made it is found that, at least on the acid side of p_H 6·0, the curves for undenatured and denatured egg-albumin are congruent. At p_H values on the alkaline side of p_H 6·0 the same is probably true but the experimental verification is less satisfactory.

The above work was carried out under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice.

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XV. THE DENATURATION OF PROTEINS.
XI. THE EFFECT OF DENATURATION ON THE CAPILLARY ACTIVITY OF THE SOLUTIONS OF CERTAIN PROTEINS.

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(*Received December 23rd, 1932.*)

It was shown by Loughlin and Lewis [1932] that on denaturation the viscosity of solutions of crystalline egg-albumin and oxyhaemoglobin increases, the increase for a solution of given strength being constant and independent of p_H provided no flocculation is allowed to occur. In the above communication, and in a subsequent one by the writer [1932] this result is interpreted to mean that the process of denaturation is accompanied by an increase in the size of the protein unit, the volume of the denatured unit being approximately twice that of the undenatured. Thus the examination of the effect of denaturation on the viscosity of the protein solutions in question leads to an important conclusion in connection with the process of denaturation itself. It is quite possible, therefore, that an analogous investigation of the effect of denaturation on the capillary activity of these proteins may also be of value in indicating the nature of the process of denaturation.

The present communication gives an account of such an investigation, which is parallel in every respect with the previous one on the viscosity, separate measurements of the capillary activity being made on undenatured solutions, and on solutions which had been denatured, but in which flocculation had not been allowed to occur even to the slightest extent.

Examination of the literature shows that once again, although a considerable number of measurements have been made using different methods, none seems to have been made previously with the specific object of showing whether any change occurs on denaturation, although Young [1922] has shown, using the Traube stalagmometer, that there is a decrease in the surface tension of solutions of egg-albumin when they are denatured by light. It is true that St Johnston [1927] concludes from his work on 0·3 % solutions of egg-albumin that denaturation causes a decrease in surface tension, but his method of obtaining this result is not very convincing, and no attempt was made to obtain separate measurements on the undenatured and denatured protein.

Experimental.

The crystalline egg-albumin was prepared by the method of Sørensen [1917], and the oxyhaemoglobin by a modification of the method of Schulz [1898]. The protein solutions were freed from salt by prolonged dialysis, and the concentration of protein in the stock solutions was estimated by the method of Devoto as modified by Hopkins [1900].

The method used for the measurement of the surface tensions of the protein solutions was the "maximum bubble pressure" method of Sugden [1922; 1924]. The bubbling apparatus and the manometer were placed in a thermostat at 25° regulated to $\pm 0{\cdot}05°$. The manometer was observed from some distance away through a telescope, by which means the pressure difference readings were obtained more accurately and the possibility of any error due to parallax was eliminated.

The apparatus was calibrated by the use of benzene, and the accuracy of the calibration was estimated by making several determinations of the surface tension of water, and comparing the values obtained with the value given in Landolt-Börnstein Tabellen.

At first some difficulty was experienced due to the frothing of the protein solutions in the bubbling apparatus, but it was found that this frothing could be prevented by adding a drop of toluene to the solution. Provided the toluene was not allowed to get inside the tubes of the bubbler where the fresh surface was formed, the surface tension measurements were unaffected. The writer tested this point to his own satisfaction, and it also agrees with the findings of St Johnston and Peard [1925].

The p_H measurements were made by the use of the improved glass electrode method of Harrison [1930].

The capillary activity of salt-free solutions of undenatured and denatured crystalline egg-albumin.

The solutions used for the determinations were 0·3 % and were prepared by diluting the requisite volume of the stock solution with dilute sulphuric acid of such a concentration as to give the required p_H. For each determination four different rates of bubbling were used on each of two different samples of the solution, so that the surface tension values given are the mean values of at least eight determinations. The values obtained were found to be independent of the bubble rate.

The measurements on the undenatured solutions were made immediately after preparation as soon as temperature equilibrium had been established in the bubbling apparatus.

In the case of the denatured solutions the extreme precautions described previously [*cf.* Loughlin and Lewis, 1932] were taken to ensure that denaturation was not accompanied by flocculation even to the slightest extent.

The pressure differences recorded were of the order 5·3 cm. alcohol, and the widest variation obtained in any reading was 0·05 cm. alcohol. The values of the surface tension obtained can therefore be guaranteed to within about $\pm 0{\cdot}3$ dyne/cm. This is an outside figure, and most of the readings can be taken as being well within this limit.

For the sake of brevity, tables of figures are not given, but the values obtained are plotted in the form of surface tension-p_H curves.

The surface tension of 0·3 % salt-free solutions of crystalline egg-albumin at 25°.

The values obtained for 0·3 % salt-free solutions of egg-albumin are plotted in Fig. 1, inspection of which indicates the following:

(1) The dynamic surface tension of egg-albumin solutions is less than the surface tension of water.

(2) In the case of 0·3 % solutions of undenatured egg-albumin, the lowering in the surface tension is small, the average decrease being only 3 % from the surface tension of water.

(3) The lowering of surface tension in 0·3 % solutions of denatured egg-albumin is much greater, of the order 8–12 %.

(4) In the range of p_H common to both curves, *viz.* from p_H 2·3 to 4·0, the curve for denatured protein lies completely below the curve for undenatured protein, *i.e.* on denaturation in this range of p_H there is a considerable decrease in the surface tension of the protein solutions.

The curve for denatured egg-albumin cannot be extended beyond p_H 4·0 on account of the occurrence of flocculation, but from its form it is almost certain that if it could be extended, it would still lie below the curve for undenatured egg-albumin. In other words, our conclusion need not be restricted to the range of p_H common to both curves, and we may conclude that when a solution of egg-albumin is denatured, there is an accompanying decrease in the surface tension of the solution.

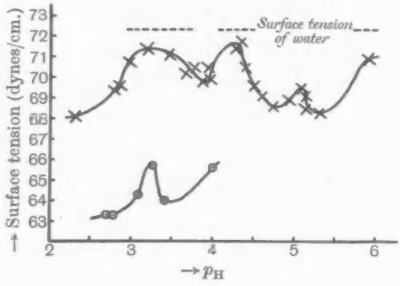

Fig. 1.

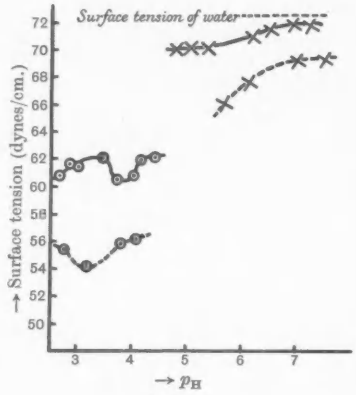

Fig. 2.

Fig. 1. Surface tension-p_H curves of 0·3 % salt-free solutions of crystalline egg-albumin at 25°.

×—× Undenatured solutions.
⊙—⊙ Denatured solutions.

Fig. 2. Surface tension-p_H curves for salt-free solutions of oxyhaemoglobin at 25°.

×—× 0·2 % undenatured.
⊙—⊙ 0·2 % denatured.
×- -× 2·0 % undenatured.
⊙- -⊙ 2·0 % denatured.

The capillary activity of salt-free solutions of undenatured and denatured oxyhaemoglobin.

For oxyhaemoglobin, two sets of determinations were made, on 0·2 and 2·0 % solutions respectively. The experimental details were exactly as described above for egg-albumin, and the results are given as before in the form of surface tension-p_H curves (Fig. 2).

The most striking feature of the curves for undenatured oxyhaemoglobin is their simplicity compared with that for undenatured egg-albumin. From Fig. 2 the following points emerge:

(1) Oxyhaemoglobin produces a decrease in the dynamic surface tension of water, thus confirming the observations of Iscovesco [1911], du Noüy [1924], and Johlin [1925].

(2) The lowering in the surface tension produced by a given concentration of oxyhaemoglobin is greater in the case of the denatured protein than in the case of the undenatured.

(3) Apart altogether from the shape of the curves, it is seen that denaturation causes a greater decrease in surface tension than does a tenfold increase in concentration.

(4) Although it is not possible to extend the curves for undenatured and denatured oxyhaemoglobin over a range of p_H common to both, it is fairly obvious, especially in the case of the 0·2 % solutions, that if extension were possible, the curve for undenatured protein would remain considerably above the curve for denatured. In other words, the behaviour of oxyhaemoglobin is analogous in this respect to that of egg-albumin, the effect of denaturing a solution of oxyhaemoglobin being to produce a decrease in its surface tension. It should be noted, however, that the decrease in surface tension on denaturation is not independent of p_H.

In passing, it is interesting to note the similarity between the surface tension-p_H curves for the dilute denatured solutions of the two proteins. In both cases, starting in the very acid region, the curve rises slightly with rise in p_H and attains a maximum at p_H 3·3 to 3·4, falling thence to a minimum at p_H 3·7 to 3·8, and finally rising once more. At the present stage it is not possible to state whether this similarity has any significance, but it is a point worthy of consideration.

For our present purpose, however, the important conclusion to be drawn from the results given above is that on denaturation there is a decrease in the surface tension of the protein solutions under consideration, although this decrease is not independent of p_H as is the case for the increase in viscosity on denaturation.

Discussion.

First of all, let us consider very briefly the results which have previously been obtained when surface tension measurements have been made on solutions of systems similar to those with which we are dealing at present. In general it is found that the various investigators have paid most attention to the behaviour at the isoelectric point. Thus, using the Sugden apparatus, Price and Lewis [1929] obtained a maximum in the surface tension-p_H curve for lecithin at the isoelectric point. Similarly, using the same method, Jones and Lewis [1932, 2] found the same result for glycine, as also did St Johnston and Peard [1925; 1926] for gelatin, and St Johnston [1927] for egg-albumin.

Reverting to the results given above, it is seen that this result is obtained for oxyhaemoglobin but not for egg-albumin. In fact, on the contrary, there is a minimum in the surface tension-p_H curve of egg-albumin at the isoelectric point. This is surprising especially in view of the findings of St Johnston. It is found, however, that other investigators also report a minimum. Thus, working with gelatin, and using a static method for measuring the surface tension, de Caro [1925] and Johlin [1925] report a minimum at the isoelectric point. Furthermore de Caro and Laporta [1929; 1930] state that not only

gelatin but also egg-albumin and serum-albumin, whether in dilute or concentrated solution, have a minimum surface tension (both static and dynamic) at the isoelectric point. Clearly, therefore, there is a serious conflict of evidence with regard to the actual behaviour of the surface tension-p_H curve at the isoelectric point.

Partly for this reason, and partly also on account of the curious periodicity in the curve for the undenatured egg-albumin, a large number of determinations were made by the author, averaging in the important range of p_H 3·6–5·6 about one determination per 0·1 change in p_H. In the writer's case, therefore, the minimum does not depend on only three or four determinations, but is clearly defined by a large number. In face of this it is difficult to believe that the minimum is unreal, so that it becomes necessary to determine whether the maximum observed by St Johnston is supported by unassailable experimental evidence.

On examining the experimental procedure of St Johnston it is found that, fearing possible complications due to the occurrence of denaturation, he endeavoured to overcome this difficulty by keeping all his albumin solutions at 25° for 24 hours before making the surface tension measurement. In doing this he defeated his own object, for as shown by P. S. Lewis [1926], the rate of denaturation of egg-albumin varies greatly with p_H, so that such treatment would produce widely different degrees of denaturation at different p_H values. Since, as we have seen above, the surface tension is diminished on denaturation St Johnston would obtain an enhanced decrease in surface tension according to the extent to which denaturation had occurred. In fact it is probable that whilst his curve in the region of p_H 6·9 should correspond to the curve for undenatured egg-albumin, in the very acid region he would be measuring the surface tensions of solutions of practically completely denatured egg-albumin. This may be gathered from the following facts.

St Johnston performed a series of experiments in which he measured the surface tensions of a number of egg-albumin solutions, firstly after the above treatment, and secondly after a further heating at 60° for 15 minutes. The solutions at p_H values above 4·0 were coagulated by this treatment, but those at p_H values more acid than 3·6 remained clear so that true surface tension measurements could be obtained. For these solutions, heating at 60° for 15 minutes is sufficient to produce practically complete denaturation of the albumin. Yet this treatment at p_H 3·10 diminished the surface tension by only 0·1 dynes/cm., at p_H 2·03 by only 0·8 dynes/cm., and so on. In view of the writer's experiments, complete denaturation would be expected to produce a much greater decrease than this. Thus it is possible to infer that denaturation had already proceeded to a considerable extent, in the solutions at p_H values $< 3{\cdot}5$ at least, before the heating to 60° had been performed. Some confirmation of this inference is given by the following comparisons. At p_H 5·3 the St Johnston value of 68·0 agrees with the value 68·3 given above for undenatured egg-albumin; similarly at p_H 4·7 the values are 68·5 and 68·6 respectively, but at p_H 2·6 St Johnston's value of 62·1 is comparable rather with the writer's value 63·3 for denatured egg-albumin.

Thus it is possible that the St Johnston maximum is due in part at least to the interfering effects of the process of denaturation of the egg-albumin, and the balance of probability is that the minimum obtained by the writer represents the true behaviour of the curve at the isoelectric point.

Since denaturation facilitates subsequent flocculation it is reasonable to assume that denaturation implies an opening up of the lines of force associated

with the groupings which are responsible for the capacity of denatured units to adhere to one another. That such release of valency should go hand in hand with an actual distension of the protein unit upon denaturation (as the viscosity results suggest) is likewise not unreasonable. The capillary activity of such denatured units is therefore to be ascribed in part to the increase in volume, but probably to a greater extent to the enhanced chemical reactivity of the unit. That no simple relation exists is evidenced by the fact that the simple amino-acids, *e.g.* glycine, though capillary active, nevertheless raise the surface tension whereas proteins lower it. That the size of the unit may play a part is at least suggested by the capillary activity (involving decrease in tension) exhibited by dispersions of lecithin, which have been shown to owe their activity in this respect to the large units, *i.e.* units large compared with molecular dimensions [Jones and Lewis, 1932, 1], whilst the decreased capillary activity when these units are diminished in size, as they presumably are when alcohol is present [Price and Lewis, 1929], is at least not at variance with a possible relation between increase in size and decrease in surface tension on denaturation.

SUMMARY.

1. Surface tension measurements have been made on solutions of undenatured egg-albumin and oxyhaemoglobin, and on deliberately denatured but unflocculated solutions of the same proteins.

2. In both cases there is a decrease in the surface tension of the solution on denaturation, although unlike the increase in viscosity, this decrease is not independent of p_H.

3. It is shown that the enhanced capillary activity is not in disagreement with the increase in size of the unit which takes place on denaturation.

The above work was performed under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice.

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XVI. THE DETERMINATION OF INORGANIC IODINE IN DESICCATED THYROID GLAND.

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(*Received December 12th, 1932.*)

In the course of a study of the iodine compounds in thyroid glands removed from patients with exophthalmic goitre after a pre-operative course of treatment with Lugol's iodine solution, it became necessary to estimate the proportion of the total iodine in the gland present as inorganic iodide. A survey of the literature gave no indication of the existence of iodine in the gland in any forms other than inorganic iodide, protein-iodine, and a small trace combined with fat and soluble in light petroleum, which could be disregarded since in no case was it found to exceed 0·1 % of the total iodine content. It was therefore assumed that an exhaustive extraction with ethyl alcohol would give a method of separation of inorganic iodide from the remainder.

The glands were finely minced immediately after removal and placed in a vacuum desiccator with P_2O_5 for 24 hours, after which they were roughly ground, replaced in the desiccator and dried to constant weight. It was then possible to grind them to a uniform, fine powder. A weighed amount of the dry powder was extracted with ethyl alcohol for 12–16 hours in a Soxhlet apparatus and the iodine estimated in the extract. By this method it was found that alcohol dissolved from normal glands an average amount of 1·5 % of the total iodine, the range for six glands being 1·35 to 1·74 %. From the pathological glands varying amounts up to 65 % were dissolved.

Kendall and Simonsen [1928] state from the results of several previous writers that the normal thyroid gland does not contain inorganic iodine. Gutman *et al.* [1932] examined 20 normal glands and found amounts varying from 3·2 to 18·5 % (average 6·4 %) of the total iodine to be present in the inorganic state. They used an aqueous extraction method proposed by Harington and Randall [1929] for estimation of inorganic iodine in commerical desiccated thyroid glands, which is dependent on the degree of denaturation of the gland produced by the drying process. When 0·2 g. of the non-heated dried gland is shaken for 2 hours with 20 cc. of distilled water, 80–90 % of the total iodine is found in the aqueous extract, and in one sample of commercial dried thyroid powder available approximately 75 % of the iodine was dissolved by one extraction. Since only a small proportion of this iodine is dialysable, it is suggested that the method is not suitable for estimation of inorganic iodides in glands which may not have become completely denatured in process of desiccation.

The following experiments were carried out with the objects of vindicating the alcoholic extraction method and finding some suitable method for rapid separation of the inorganic iodine.

Experimental.

Effect of drying temperature on solubility of iodine compounds in the gland.

A normal thyroid was finely minced and divided into three portions which were separately dried to constant weight; A in vacuum desiccator with P_2O_5 without heat; B in an oven at 65°, and C at 110°. The results of extraction of these and of a commercial preparation with water and with alcohol, and of dialysis of the aqueous extracts through cellophane are given in Table I.

Table I.

Glands	A	B	C	Commercial
Total iodine, mg. per 100 g.	189	194	194	186
Alcoholic extract, % of total iodine	1·11	1·33	3·30	3·24
Aqueous extract, % of total iodine	83·5	2·8	9·0	81·0
Dialysis of aqueous extract from	174 mg.	291 mg.	274 mg.	187 mg.
Iodine in dialysate after 16 hours, in γ	5·6	5·4	26·2	4·9
+24 ,,	2·7	2·2	8·0	2·6
+48 ,,	1·2	0·7	2·9	1·0
Total	9·5	8·3	37·1	8·5
Total as % of I in gland	2·9	1·5	7·0	2·4

Table II gives the results of similar experiments on three glands from patients with exophthalmic goitre treated before operation with Lugol's iodine solution. These glands were dried without application of heat.

Table II.

Glands	W.H.	L.M.	E.C.
Total iodine, mg. per 100 g.	210	175	181
Alcoholic extract, % of total iodine	14·5	40	65
Aqueous extract, % of total iodine	81	90	75
Dialysis of aqueous extract from	333 mg.	280 mg.	237 mg.
Iodine in dialysate after 16 hours, in γ	30·4	71	89
+24 ,,	10·2	21·2	37
+48 ,,	3·8	5·8	16
+48 ,,	2·1	3·2	8·2
Total	46·5	101·2	150·2
Total as % of I in gland	10·8	31·5	45·5

Dialysis of aqueous "suspension" of glands.

A small amount of the dried powder (about 0·2 g.) was shaken with 20 cc. cold distilled water for 2 hours. At the end of this time approximately 75 % of the solid had dissolved, and the whole solution, together with the suspended, undissolved tissue was placed in a cellophane tube suspended in running water for 4 days. The contents of the tube were washed out and the amount of non-

Table III.

Gland	Iodine in alcoholic extract % of total	Result of dialysis
L.M. "iodised"	40·0	212 mg. gland = 0·371 mg. I after 112 hours contained 0·206 mg. I. Loss = 44 %
W.H. "iodised"	14·5	181 mg. gland = 0·38 mg. I after 88 hours contained 0·323 mg. I. Loss = 15 %
Normal 114	1·8	165 mg. gland = 0·205 mg. I after 112 hours contained 0·198 mg. I. Loss = 3·4 %

dialysable iodine was estimated. It was found that the percentage of the total iodine lost during dialysis was approximately equal to that removed by exhaustive extraction with ethyl alcohol.

Extraction of desiccated gland with cold alcohols.

Shaking with cold ethyl alcohol for 2–3 hours as in the aqueous extraction process dissolved out much smaller amounts of the total iodine than extraction in the Soxhlet apparatus (only 1/10 to 1/3 of the amount). Since methyl alcohol is a very much better solvent for iodides than ethyl alcohol, experiments were made with this solvent, and it was found that a single extraction in the cold dissolved out approximately the same proportion of the total iodine in the gland as that dissolved by extraction for 16 hours with ethyl alcohol in a Soxhlet apparatus (Table IV).

Table IV.

Gland	Iodine extracted by C_2H_5OH in Soxhlet mg. per 100 g.	Iodine extracted by cold CH_3OH mg. per 100 g.
Normal	2·10	1·95
,,	2·57	2·17
,,	2·84	2·64
,,	1·80	1·70
,,	1·82	1·88
,,	6·3	6·3
Commercial	6·0	5·5
Exophthalmic goitre: "iodised"	70·0	68·4
,,	117·0	113·0
,,	30·4	28·6

A weighed portion of the powdered gland (0·2–0·3 g.) was introduced into a stoppered tube and shaken for 2½ hours with 20 cc. cold methyl alcohol. The contents of the tube were then filtered and the tube and powder washed with a little methyl alcohol. This extract was evaporated and its iodine content estimated in the usual manner.

Finally, methyl alcoholic extracts from larger amounts of glands were made and evaporated almost to dryness. The residues were dissolved in distilled water, and, after estimation of iodine in an aliquot part of the solution, dialysed in cellophane tubes suspended in running water. After about 4 days the iodine remaining in the tubes was estimated. It was found that in every case 94 to 99 % of the iodine had passed through the membrane.

This methyl alcoholic extraction is, therefore, suggested as a method for determination of inorganic iodide in desiccated thyroid gland, especially for commercial products where the method of drying the gland is unknown.

The estimations of iodine were made by von Fellenberg's method, of which a very full description is given by Lunde *et al.* [1929]. Estimations were made in duplicate in practically every case.

Summary.

1. Extraction of desiccated thyroid glands with water dissolves iodine compounds other than inorganic iodides in amounts which depend on the degree of denaturation of the gland. Aqueous extraction is, therefore, not suitable as a method of estimation of inorganic iodine.

2. Exhaustive extraction with alcohol removes only dialysable iodine, together with a trace of iodine combined with fat.

3. A rapid method is described whereby inorganic iodides can be estimated in desiccated thyroid glands regardless of the method by which the glands were dried.

The author wishes to express his thanks to Prof. E. C. Dodds for helpful criticism.

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XVII. A NOTE ON THE USE OF ALCOHOL AS A SUGAR EXTRACTANT IN PLANT TISSUE ANALYSIS.

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(*Received December 12th, 1932.*)

In the estimation of the sugar present in plant material ethyl alcohol has been and still is very largely used as an extractant. It has the advantage of not extracting starch or pectin from the material, it can be rapidly removed by evaporation when the extract has been prepared; further, enzyme action is rapidly inhibited during the preliminary alcohol treatment which also ensures that the extract remains sterile.

Barton-Wright and Pratt [1930] however seriously questioned the suitability of alcohol as an extractant and showed that with fairly large (50 g.) samples of leaf material alcoholic extraction may lead to high results for hexoses as compared with aqueous extraction, possibly owing to oxidation of a certain amount of the alcohol to aldehydes.

As the present author desired to estimate the sugars present in relatively small amounts of leaf material containing, in addition to sugars, starch, hemicelluloses and dextrins which may become partially hydrolysed during sucrose hydrolysis, it was decided to investigate the use of alcohol as an extractant for quantities of leaf material of 25 g. and less.

Experimental.

Material used and method of sampling.

The leaves of *Hymenocallus* sp., *Tropaeolum majus* and *Hedera helix* were used. The leaves of these three plants vary considerably in their texture and apparent chlorophyll content; also the last contains considerably more sugar than the two former types.

About 200 g. of the leaf, in the case of *Hymenocallus* and 50 g. in the case of *Tropaeolum*, were placed, immediately after gathering, in beakers in a cold chamber at $-18°$ to $-20°$ for about 24 hours. The whole of each batch was ground (in the cold chamber) in a small coffee mill. The ground material was then thoroughly mixed to ensure uniformity throughout the sample.

About 25 g. lots in the case of *Hymenocallus* and 6–8 g. lots in the case of *Tropaeolum* were then weighed into separate beakers, still in the cold chamber; these lots were transferred to the laboratory and the sugars immediately extracted and estimated by the methods to be described later. In certain cases small amounts of pure dry sugars (glucose and sucrose) were added to the material to determine the "percentage recovery" of sugars during extraction. When this was done the sugar was weighed into the beaker before placing the latter in the cold chamber previous to the addition of the frozen leaf powder.

The final results are expressed as a percentage of the fresh weight of the material, fresh weight here meaning the weight of frozen powder taken for extraction. Admittedly this is not the true fresh weight of material, but as the same method is used for both alcohol and water extractions the results do give the true "relative" sugar percentages; this is all that is required for the present work.

In the preparation of the ivy leaf material about 10–12 g. of leaves were taken and each leaf cut into two down the mid-rib. Each set of halves was weighed separately, frozen at $-18°$ for 24 hours and then ground (at $-18°$) in a small coffee mill, washing and removing all the leaf material from the mill with washed silver sand. The two portions of ground material were extracted, one with alcohol and the other with water. The sugar results are expressed as percentage of the fresh weight of the halves before freezing.

Methods of alcoholic and of aqueous extraction.

Alcoholic extraction[1]. The ground leaf material was transferred, immediately on removal from the cold chamber, small quantities at a time, to 200 cc. of boiling alcohol of sufficient concentration to bring the final alcohol concentration to approximately 80 % after allowing for the moisture present in the leaf material. Any powder adhering to the sides of the beakers was washed into the flask containing the alcohol with a few cc. of hot 80 % alcohol. The liquid was boiled under a reflux condenser for half an hour and then filtered through a fluted, hardened filter-paper and the paper and contents, after washing with hot 80 % alcohol, transferred to a hot extractor and extracted on a boiling water-bath for 5 hours. By this time the alcohol dropping from the leaf material was colourless. The alcohol was evaporated from the combined extracts under diminished pressure at 40–45°. The residue was taken up with hot distilled water, any material adhering to the distillation flask being removed by shaking with washed silver sand. Lead acetate was added to this aqueous extract which had p_H approximately 6·0. The precipitate was allowed to settle for one hour, filtered off and well washed with warm water; the excess lead was removed from the filtrate with Na_2HPO_4. The precipitate was allowed to settle overnight, filtered off and well washed, the filtrate and washings being made up to 250 cc.; aliquot parts were taken for the sugar determinations.

Aqueous extraction. The weighed and ground material was transferred as described above to 300 cc. of boiling water and the liquid carefully boiled (to avoid violent frothing) for 10 minutes. The liquid was then filtered through a thoroughly washed fine muslin bag fitted into a large Gooch adapter, the liquid being finally pressed from the residue with a pestle. A short length of rubber tubing fitted with a pinch-cock was placed on the lower end of the adapter and 50–60 cc. of distilled water poured over the residue in the bag. After allowing the bag and contents to soak for 2 hours the pinch-cock was opened and the extract allowed to run into the vessel containing the first extract. This extraction with 60 cc. of distilled water was repeated three further times and then the combined extracts were evaporated to a small volume under diminished pressure at 45°. The concentrated extract was cleared with lead acetate, *etc.* exactly as in the case of the alcoholic extraction, and was finally brought to 250 cc.

[1] The alcohol used was prepared by dilution of rectified spirit (approximately 97 % ethyl alcohol) with distilled water. The residue obtained on evaporation to dryness under diminished pressure at 45° was free from aldehydes.

In the case of *Tropaeolum* and ivy, since smaller amounts of material were taken, the volumes of alcohol and of water used in the extractions were about half those stated above; in all cases the final volume of the cleared extract was 250 cc. No ammonia or other alkali was added to the alcohol or water used in the extractions, since previous experiments had shown that with and without addition of ammonia (1·5 cc. of sp. gr. 0·880 per 100 cc.) to the extractants the hexose and total sugar contents of the extracts were the same, *i.e.* no hydrolysis of sucrose (where present) had taken place during extraction; this was to be expected, the p_H of the extracts being about 6·0.

Further extraction of the leaf residues with hot 80 % alcohol after both alcoholic and aqueous extractions failed to remove any further reducing substances showing that the methods of extraction used gave complete extraction of sugars.

Methods of sugar determination.

In the case of *Hymenocallus* leaves the sugars were estimated by the Shaffer-Hartman copper method [1920], the "combined" reagent being used. It was found necessary to re-standardise this method, for although the conditions used were those recommended by Shaffer and Hartman the calibration curve for glucose was found to be appreciably different from that given by these authors. The Hanes[1] [1929] ferricyanide micro-method was also used after suitable dilution of aliquot parts of the cleared extracts. In both cases the reducing sugars were expressed as glucose as also were the total sugars obtained after hydrolysis with 10 % citric acid; in the latter case due allowance had to be made for the "blank" reading given by citric acid alone. The results obtained by the Shaffer-Hartman method cannot be strictly compared with those obtained by the Hanes method for it was found later that fructose, glucose and invert sugar have somewhat different relative reducing powers towards the copper and the ferricyanide reagents. This does not, however, invalidate accurate comparisons between the alcoholic and aqueous extractions provided the results are obtained for both methods of extraction by the same methods of estimation. In the cases of *Tropaeolum* and ivy where 6–8 g. samples were taken and consequently relatively small amounts of sugar were present in the extracts it was only possible to use the Hanes method.

The results obtained are given in Tables I and II. Duplicate extractions were carried out in all cases and the results given are the mean of two sugar estimations on each of two separate extractions, the mean that is of four similar

Table I.

	Alcoholic extraction		Aqueous extraction	
	Shaffer-Hartman method	Hanes method	Shaffer-Hartman method	Hanes method
Hymenocallus sp.				
% hexose	1·75	1·73	1·70	1·76
% added glucose recovered	95·0	99·5	97·0	100·0
% total sugars (as hexose)	1·76	1·93	1·71	2·04
% added sucrose recovered	—	100·0	—	100·0
Tropaeolum majus				
% hexose	—	1·40	—	1·31
% total sugars (as hexose)	—	2·55	—	2·58

[1] The modified Hanes method suggested by Hulme and Narain [1931] had not been evolved at this time.

Table II.

Leaf batch (*Hedera helix*)	Alcoholic extraction		Aqueous extraction	
	% hexose	% total sugar (as hexose)	% hexose	% total sugar (as hexose)
II	3·01	4·16	3·28	4·17
III	4·08	4·36	4·08	4·24

estimations. Where "percentage recovery" of sugar is given the recovery is based on separate extractions with and without the addition of known quantities of the sugars concerned.

The solutions (from both alcoholic and aqueous extractions) in which the leaf sugars were estimated, gave negative reactions for acetaldehyde and formaldehyde with Schiff's test and the dimedon reagent used by Barton-Wright and Pratt [1930].

Discussion.

Although the agreement between the results for aqueous and alcoholic extraction is not exact there is no marked difference in any one direction. In view of recent work by Maskell and Narain (still unpublished) the explanation of the high figures for total sugars given by the Hanes method as compared with those obtained by the Shaffer-Hartman method lies, in all probability, in the fact that certain substances present in many leaves yield reducing products on acid hydrolysis which have a higher reducing action towards the ferricyanide than towards the copper reagent. This again does not affect the point at issue.

To investigate further the possibility of the formation of aldehydes from the interaction of alcohol and chlorophyll during alcoholic extraction of leaf material, 20 g. of frozen and ground tree ivy leaves were boiled with 80 % alcohol for 10 hours—a considerably more drastic treatment than that normally used for sugar extraction; a dark green solution resulted indicating the presence of a large amount of chlorophyll. After filtering off the leaf residue the alcohol was evaporated under diminished pressure at 50° the residue taken up with water and the aqueous extract cleared in the usual way. The Schiff test gave only a faint coloration; a small amount (barely sufficient when dry, for a melting-point determination) of aldomedon compound was obtained from 200 cc. of the extract using the technique of Klein and Werner [1926]. The product had M.P. about 135° and was therefore presumably acetaldomedon (Klein and Werner give the melting-point of pure acetaldomedon as 139°). It is therefore clear that, even under more drastic conditions of alcoholic extraction than those normally used, little more than a trace of acetaldehyde is present in the final extracts on which the sugar determinations are made. It is in any case unlikely that volatile aldehydes would remain behind after evaporation of the alcohol unless some form of combination occurs resulting in the formation of a non-volatile compound.

Hence the alcoholic method of quantitative sugar extraction may be considered to be free from appreciable inaccuracies due to the formation of reducing substances during extraction, at least so far as relatively small leaf samples (25 g. or less) are concerned.

Summary.

By the estimation of the sugars extracted from the leaves of three types of plant by hot alcohol and by cold water it is shown that, in the cases examined, no appreciable error is introduced during alcoholic extraction through the formation of aldehydes.

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XVIII. THE RELATIVE VALUE OF CLINICAL TESTS FOR BLOOD.

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Most of the clinical tests for blood are purely qualitative. Slight variations in technique may affect considerably the sensitivity of some of the reactions; it is therefore important to describe the technique. In work previously published, the results have often been expressed in terms of dilutions of blood; such blood was presumably from a healthy individual, but even then its haemoglobin content may have varied between limits of the order of 12 to 16 g. per 100 cc. It is well recognised that some derivatives of haemoglobin are more readily detected spectroscopically than others, but no quantitative data have been encountered on the relative sensitivity of such methods.

In the present investigation an attempt has been made to place these clinical tests on a quantitative basis, and so to establish their relative values. The limits of sensitivity of spectroscopic tests for haemoglobin and its derivatives (mainly in aqueous solution) were first established (see Table I). Similar measurements were next obtained when haemoglobin and its appropriate derivatives were in solution in blood, urine or faeces. Finally the results of the spectroscopic tests were compared with certain of the purely chemical reactions applied to the same fluids. To save repetition it should be noted that wherever haemoglobin derivatives have been expressed as mg. or g. per 100 cc., these results are calculated in terms of oxyhaemoglobin.

Spectroscopic tests.

Solutions of oxyhaemoglobin and its derivatives were diluted until their spectra were only just visible under the standard conditions employed. The dilution being known, and the initial concentration of the oxyhaemoglobin solution from which the derivatives were prepared having been found by a determination of the oxygen-combining power, the limits of sensitivity for the detection of the different derivatives were calculated in terms of oxyhaemoglobin mg. per 100 cc. Clearly the results are of relative value only; other observers might differ in their appreciation of the point at which the spectra were only just visible; that does not matter. Variations in the writer's own definition of the end-point from time to time are of importance, and of necessity make the comparisons approximate only; at the same time the results (see Table I) were more consistent than had been expected beforehand, so much so that in the writer's opinion they are of definite value for comparative purposes.

A summary follows of the procedure employed.

Fresh blood, obtained from an arm vein, was used; some degree of constriction of the vein was generally necessary, thus accounting for the rather high percentage of haemoglobin in many instances. The blood was oxalated, suitably treated, diluted, and viewed with the same direct vision spectroscope in each

experiment. The solutions were all placed in boiling-tubes of 1″ bore, and viewed under identical conditions of lighting, with a 60-watt frosted bulb.

In each case the oxygen capacity of the fully-aerated blood was determined in a Van Slyke volumetric apparatus, 1·9 cc. being deducted to allow for oxygen and nitrogen in physical solution, and the resulting oxygen-combining power (cc. O_2 per 100 cc.) was converted into haemoglobin (g. per 100 cc.) by multiplying by 0·746.

Subsequent treatment was as follows.

Oxyhaemoglobin. 1·0 cc. of blood was diluted with distilled water, until first the β band and then the α band was just visible.

Carboxyhaemoglobin. A 1 in 50 aqueous solution of blood was saturated with coal gas, suitably diluted with water, and examined at once.

Sulphaemoglobin. To 5 cc. of 1 in 50 blood, 0·9 cc. of 0·1 % phenylhydrazine hydrochloride and 0·1 cc. of water saturated with H_2S [Van den Bergh and Wieringa, 1925] were added; the mixture was suitably diluted and examined immediately.

Reduced haemoglobin. 10 cc. of 1 in 50 blood were treated with a knife-point of solid $Na_2S_2O_4$, and subsequently diluted.

Methaemoglobin. 1 cc. of blood was laked in about 20 cc. of distilled water, 1 cc. of 4 % potassium ferricyanide was added, and the volume made up to 50 cc. [McEllroy, 1920]. After standing for a few minutes, the mixture was further diluted. No observations were made for the δ band.

Acid haematin. 1 cc. of blood was diluted to 25 cc. with 0·1 *N* HCl, mixed, and allowed to stand for at least 15 minutes. The mixture was further diluted with water to determine the limits for the α, β and γ bands. No attempt was made to define the limit for the δ band.

Alkaline haematin. To 5 cc. of the above 1 in 25 acid haematin solution 5 cc. of 5 % sodium hydroxide were added. The mixture was heated, but not to the boiling-point, and then suitably diluted with water.

Haemochromogen. 1 cc. of the above 1 in 50 alkaline haematin solution was treated with a knife-point of solid sodium hydrosulphite, and suitably diluted.

Acid porphyrin. 0·1 cc. of blood was added drop by drop to 9·9 cc. of concentrated sulphuric acid, shaking well throughout; the blood was not allowed to fall directly on to the wall of the tube, and the mixture was not examined until all the blood had gone into solution; sometimes several attempts had to be made before complete solution was attained. Further dilution was made with the concentrated acid.

Alkaline porphyrin. A simple method for producing quantitatively alkaline porphyrin directly from blood is not available. The difficulty was overcome by preparing an alkaline porphyrin solution of unknown concentration from an acid porphyrin solution, and then diluting it till the absorption bands were only just visible. The final solution of alkaline porphyrin was then converted into acid porphyrin and further diluted to the limits for the acid porphyrin bands. Knowing the dilutions at the different stages, the concentration of the initial solution of alkaline porphyrin was calculated.

The initial alkaline porphyrin solution was prepared as follows. About 1 cc. of blood was added to 10 cc. of concentrated sulphuric acid, mixed thoroughly, and poured cautiously into about 350 cc. of cold distilled water. Of the resulting precipitate, that portion which floated on the surface was removed with a spoon-shaped glass spatula, treated with concentrated ammonia (sp. gr. 0·880), and filtered. The filtrate was further diluted with concentrated ammonia till the α band was only just visible.

Table I. *Limits of sensitivity for spectroscopical detection of haemoglobin and its derivatives (solutions 1" thick).*

The figures represent the concentration of pigment at the limiting dilution expressed as mg. oxyhaemoglobin per 100 cc.

Blood No.		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII		
Haemoglobin g. per 100 cc. of untreated blood...		16·8	16·7	17·8	14·2	14·3	14·9	9·3	16·7	18·9	18·0	14·2	17·5	Average	Colour of solution when diluted to spectroscopic limit
Oxyhaemoglobin	α	4·9	4·5	4·6	4·4	5·0	5·4	5·8	5·6	4·7	4·5	5·1	5·4	**5·0**	Very pale straw
	β	8·4	8·4	8·9	8·9	8·7	8·5	8·5	8·8	9·0	9·0	8·6	9·2	**8·7**	
Carboxyhaemoglobin	α	7·6	7·4	7·4	7·1	8·7	8·5	8·1	8·0	8·4	7·8	7·9	7·8	**7·9**	Pale pinkish-straw
	β	10·5	11·1	11·1	9·5	13·6	11·9	11·6	11·1	10·2	11·3	10·9	11·3	**11·2**	
Sulphaemoglobin	α	48·0	47·7	59·3	40·6	51·1	49·7	44·3	55·7	50·4	51·4	54·6	58·3	**50·9**	Greenish-yellow-brown
	β	12·2	13·9	14·2	12·9	13·6	12·4	11·6	17·6	14·5	15·0	15·8	17·5	**14·3**	
	γ	25·9	27·8	32·4	28·4	31·8	29·8	37·2	39·3	32·9	30·0	31·6	38·9	**32·2**	
Reduced haemoglobin		48·0	47·7	50·9	40·6	49·3	43·8	46·5	51·4	47·3	51·4	47·3	50·0	**47·7**	Pale red-brown with green tint
Methaemoglobin	α	56·0	55·7	61·4	56·8	57·2	54·2	51·7	59·6	54·0	60·0	56·8	63·6	**57·3**	Well marked yellow-brown
	β	96·0	92·8	101·7	94·7	102·1	99·3	93·0	111·3	108·0	112·5	97·9	116·7	**102·2**	
	γ	67·2	66·8	79·1	71·0	75·3	74·5	71·5	72·6	84·0	72·0	71·0	77·8	**73·6**	
Acid haematin	α	67·2	74·2	71·2	56·8	65·0	66·2	62·0	66·8	68·7	69·2	63·1	70·0	**66·7**	Well marked brown
	β	224·0	222·7	237·3	189·4	238·3	198·7	232·5	222·7	189·0	211·8	236·7	233·3	**219·7**	
	γ	84·0	83·5	79·1	71·0	79·5	85·1	71·5	83·5	84·0	81·8	71·0	76·1	**79·2**	
Alkaline haematin		152·7	167·0	178·0	142·0	168·2	165·6	155·0	167·0	171·8	180·0	157·8	175·0	**165·0**	Well marked greenish-brown
Haemochromogen	α	2·4	3·3	3·9	2·8	2·9	3·0	3·1	3·0	2·5	2·6	2·8	2·5	**2·9**	Pale straw
	β	21·0	20·9	19·8	18·9	17·9	18·6	19·0	18·6	21·0	20·0	20·3	23·3	**19·9**	
Acid porphyrin	α	33·6	33·4	35·6	31·6	35·8	37·3	37·2	39·3	34·4	36·0	35·5	38·9	**35·7**	Pale greenish-plum
	β	10·5	10·4	11·1	8·9	9·9	10·3	10·6	11·1	11·3	12·0	10·9	11·7	**10·7**	
Alkaline porphyrin	α	—	—	—	—	—	—	77·2	76·0	77·8	80·1	72·2	77·6	**76·8**	Well marked dirty brown
	β	—	—	—	—	—	—	38·2	36·9	37·6	39·8	35·1	35·0	**37·1**	
	γ	—	—	—	—	—	—	25·7	25·3	25·9	26·7	24·1	25·9	**25·6**	

The results for alkaline porphyrin were arrived at indirectly (see text).

To 10 cc. of the above final solution of alkaline porphyrin concentrated sulphuric acid was added with great care, cooling between each addition, until the acid was in excess. The mixture was then further diluted with the concentrated acid until the α band of acid porphyrin was only just visible.

Having thus obtained the spectroscopic limit for the α band of alkaline porphyrin, the limits for the β and γ bands were defined by noting the dilutions subsequently required to make each in turn only just visible. No observations were made on the limit for the δ band.

Twelve samples of blood with haemoglobin contents varying between 9·3 and 18·9 g. per 100 cc. were investigated. The results are given in Table I.

Tests for abnormal haemoglobin derivatives in whole blood.

Clinically the blood may be examined for CO-haemoglobin, methaemoglobin, or sulphaemoglobin. It is impossible to apply the data obtained in the above section directly to such tests, because in blood a relatively small proportion of the abnormal derivative of haemoglobin has to be detected in the presence of a relatively large proportion of oxyhaemoglobin. A certain initial dilution of the blood is unavoidable solely to remove the interference by oxyhaemoglobin. Two boiling-tubes (1″ diameter) were placed in line between the light and the spectroscope. In one the blood was diluted just sufficiently to remove the interfering action of oxyhaemoglobin. In the other the haemoglobin derivative was diluted to such an extent that its absorption band could just be detected.

Methaemoglobin. In order to obtain complete laking, and a sufficiently clear portion in the red part of the spectrum to see the α band of methaemoglobin, most bloods must be diluted at least 1 in 5 with water. Occasionally 1 cc. of blood can be treated with as little as 3 cc. of water, but as a routine the dilution 1 in 5 is recommended. In the experiments therefore 1 in 5 blood was placed in one boiling-tube, and the limit for methaemoglobin viewed through this 1 in 5 blood was determined. The methaemoglobin was prepared as before; the limit of detection varied between 56 and 61 mg./100 cc. In other words methaemoglobin can be detected in 1 in 5 blood as readily as in water. Taking 60 mg./100 cc. as a safe limit, the original undiluted blood would have to contain about 0·3 g. methaemoglobin per 100 cc. before it could be detected spectroscopically.

It is reasonable to assume that the limit for the detection of methaemoglobin in plasma or serum would be about the same as in water (see Table I).

Sulphaemoglobin. Experiments on this derivative were carried out on the same lines as for methaemoglobin. Owing to the fact that the α band of sulphaemoglobin is a little nearer to the blue end of the spectrum than the α band of methaemoglobin, it was necessary for a slightly larger amount of the red portion of the spectrum to be visible. From a series of observations with varying dilutions, it is recommended that the blood be diluted 1 in 10 as a routine. Using this dilution in one of the boiling-tubes, the limit for the sulphaemoglobin in the other tube varied between 50·8 and 52·5 mg./100 cc. On this basis about 0·52 g. sulphaemoglobin per 100 cc. whole blood can be detected spectroscopically. Again it is reasonable to assume that in plasma or serum sulphaemoglobin would be detected as readily as in water (see Table I).

CO-haemoglobin cannot be detected in the presence of oxyhaemoglobin by the simple direct vision spectroscope. It was interesting, however, to obtain some idea of the concentration of CO-haemoglobin which can be detected by Hartridge's reversion spectroscope [Hartridge, 1912; 1922; Frederick, 1931]. The tests were carried out with two 1″ boiling-tubes as before. Hartridge states that the blood must first of all be diluted so that the distance between the

α and β bands is approximately equal to the width of the α band, because under these conditions slight shifts of the bands due to the CO-haemoglobin are best appreciated; this was confirmed. Using a solution of blood 1″ in thickness the necessary dilution was of the order of 1/200, though of course a lower dilution could be used if the total haemoglobin of the blood was considerably reduced. Using 1/200 blood as a routine in the one boiling-tube, it was found that 5·4 to 5·8 mg. of CO-haemoglobin per 100 cc. in the other boiling-tube gave a definite shift of the micrometer reading, using Hartridge's quantitative instrument and a dark room. It is important to realise, however, that this is not accompanied by a shift of the absorption bands of such an extent as to be clearly detectable by the unaided eye. If the instrument is used by daylight, and a clearly marked shift of the absorption bands is taken as the criterion for the presence of CO-haemoglobin (and this is a not uncommon practice clinically) the instrument is much less delicate. The writer's results using this second technique varied between 35·2 and 36 mg./100 cc. CO-haemoglobin. In other words using the proper technique about 1·1 g. CO-haemoglobin per 100 cc. can be detected with the reversion spectroscope, or if the average normal haemoglobin be taken as 14 g. per 100 cc. about 8 % of the haemoglobin must be in the form of CO-haemoglobin. With the second technique there must be about 7·2 g. CO-haemoglobin per 100 cc. before it can be detected, or about 50 % of the haemoglobin must be in the form of CO-haemoglobin.

Tests for blood in urine.

Clinical observations are concerned with haemoglobinuria, haematuria, methaemoglobinuria, and porphyrinuria.

Haemoglobinuria. Known volumes of blood, of which the haemoglobin contents were estimated, were laked with the minimum of distilled water, diluted with normal urine, and examined spectroscopically in the 1″ boiling-tubes. Simple aqueous solutions of oxyhaemoglobin diluted to the spectroscopic limit, are almost colourless (see Table I). The pigments of urine interfered slightly with the sensitivity of the spectroscopic test for oxyhaemoglobin in solution in urine. The limits obtained for urine (both α and β bands visible) varied between 8·9 and 9·6 mg. per 100 cc. as against 8·4 to 9·2 mg. for oxyhaemoglobin in water.

It was found that the phenolphthalin and the pyramidone tests (for technique see below) were a little more sensitive than the spectroscopic test, about 3·5 mg. of oxyhaemoglobin per 100 cc. of urine being detected by the phenolphthalin reaction, and about 3·4 mg. by the pyramidone method.

Haematuria. As would be anticipated, haemoglobin confined within red cells was less readily detected by the spectroscope than oxyhaemoglobin in solution in urine. For the observations unlaked blood was diluted with normal urine, mixed thoroughly and examined in the 1″ boiling-tubes. The spectroscopic limit (α and β bands visible) varied from 11·0 to 12·0 mg. oxyhaemoglobin per 100 cc. urine.

Chemical tests are definitely more sensitive for haematuria, as would be expected because the red cells are of necessity broken down. Since the sensitivity may vary considerably with alteration in the details of the technique, it is necessary to outline the methods used.

In the phenolphthalin test, 1 volume of the freshly-made and filtered Kastle-Meyer reagent [see Harrison, 1930, p. 483] is mixed with 1 volume of 3 % H_2O_2, and urine is run in, a few drops at a time, up to a total of 2 volumes, shaking

after each addition and noting whether a pink colour develops or not. The Kastle-Meyer reagent is more sensitive when freshly prepared. If it is not filtered until it is absolutely clear and free from zinc dust, the reaction for blood is less delicate. Using the above technique, haematuria was detectable when the urine contained 3·5 mg. or more of oxyhaemoglobin per 100 cc.

In the pyramidone ring test 1 volume of urine was acidified with 33 % acetic acid, and 1 volume of 5 % pyramidone in absolute alcohol was carefully superimposed. 3 % hydrogen peroxide was added, a few drops at a time, up to a total of 1 volume, allowing each drop to fall well into the solution. A lilac or mauve ring indicates a positive reaction. By this means haematuria was detected when the urine contained 3·4 mg. or more of oxyhaemoglobin per 100 cc.

By far the most satisfactory test for haematuria is the macroscopical and microscopical examination of the centrifuged deposit [Harrison, 1930, p. 17]. When 10 cc. of urine were centrifuged, red cells were obvious to the naked eye at the bottom of the centrifuge-tube when the urine contained about 0·3 mg. of oxyhaemoglobin per 100 cc. Red cells were easily found under the microscope (2 to 4 cells per field using a No. 4 eyepiece and a 1/6″ objective) when the deposit from 10 cc. was examined, and the urine contained 0·024 mg. oxyhaemoglobin per 100 cc. In other words, as usually performed, the microscopical test is more than 100 times as sensitive as the chemical tests, and more than 400 times as sensitive as the spectroscopic test for haematuria.

Methaemoglobinuria and porphyrinuria. The spectroscopic limits for methaemoglobin and porphyrin in urine were found to be substantially the same as for solutions of the same pigments in water. In clinical work however it is usually unsafe to diagnose the presence of these pigments until each and all of the characteristic absorption bands are evident. In other words the practical limits for spectroscopic examination of urine are those of the least intense bands. Methaemoglobinuria can therefore be detected (using a 1″ thickness of urine) when about 0·11 g. or more is present in 100 cc. urine (see Table I, methaemoglobin β band). Similarly porphyrinuria (alkaline porphyrin) is detectable by direct spectroscopical examination with about 0·08 g. per 100 cc. of urine (see Table I, alkaline porphyrin α band). Clinically however it is well recognised that the urine from a case of porphyrinuria may show only an atypical two-banded spectrum. This is due to the concentration of porphyrin being such that the β and γ bands but not the α band of alkaline porphyrin are visible. Using the technique employed in this investigation, the two-banded spectrum will be evident when the concentration of porphyrin lies between about 0·04 and 0·08 g. per 100 cc. of urine (see Table I, alkaline porphyrin α and β bands respectively). Lastly in clinical work it is a common practice to concentrate the alkaline porphyrin by acidifying about 10 cc. of urine with glacial acetic acid and extracting with about 3 cc. of amyl alcohol; the extract is separated and treated with one or two drops of concentrated HCl, thereby converting alkaline porphyrin into acid porphyrin, the bands of which are about twice as intense as those of the alkaline pigment (see Table I). The nett result of this technique is to make the test about six times more delicate than the direct spectroscopic examination of the untreated urine. In other words using a 1″ thickness of urine, porphyrin can be detected when the original urine contains about 0·013 g. per 100 cc.

Tests for blood in faeces.

When haemorrhage occurs low down in the intestinal canal, red cells are observed when a suspension of the faeces in 0·65 % NaCl[1] is examined microscopically. The limit of sensitivity for this microscopical test was determined approximately as follows. First the dilution necessary to prepare a suitable faecal suspension was noted; 1 g. of normal faeces required about 20 cc. of 0·65 % NaCl. Then varying amounts of normal blood were incorporated in the 1 in 20 dilution of faeces, and the amount of blood which could be readily detected was noted (2 to 4 cells per field using a No. 4 eyepiece and 1/6″ objective). For example 1 g. of faeces, 4 cc. of 1 in 500 blood (haemoglobin content = 15·0 %) in 0·65 % NaCl and 15 cc. of 0·65 % NaCl was a suitable preparation; this corresponds to 120 mg. haemoglobin per 100 g. untreated faeces.

When the bleeding is low in the gut a suspension of faeces in water, cleared by centrifuging, shows the bands of oxyhaemoglobin when examined spectroscopically. If too concentrated an extract of faeces is made, it is impossible to see through it; using the technique already outlined a suspension of 1 g. of faeces in 40 cc. was generally suitable after centrifuging. In this dilution, blood could be detected in faeces to the same degree as in water, *viz.* about 9 mg./100 cc. (corresponding to β band of oxyhaemoglobin—see Table I). That is 360 mg. of oxyhaemoglobin can be detected per 100 g. of fresh faeces.

The sensitivity of the benzidine test was defined by adding varying quantities of blood to the faeces of a healthy individual who had been on a haemoglobin-free diet for 3 days; the faeces alone gave a negative reaction. Thus 1 g. of faeces was mixed with 8 cc. of water *plus* 1 cc. of blood diluted to varying degrees with water; the dilution and the haemoglobin content of the blood were measured. The resulting suspension was boiled, cooled, and made up to 10 cc. again. To 1 volume of a saturated solution of benzidine in glacial acetic acid, 1 volume of 3 % H_2O_2 was added, and the mixture was observed for a few minutes to make sure that a blue colour did not develop, and that therefore the tube was clean. The faecal suspension was then added a few drops at a time up to a total of 2 volumes. The minimum amount of blood giving a positive reaction was noted. The limit with this technique varied from 1·1 to 2·0 mg. oxyhaemoglobin per 100 g. of faeces. Mr J. F. Barrett has informed the writer that the benzidine test is less sensitive for blood if the diet contains green vegetables, and has demonstrated that the addition of a watery extract of cabbage will turn a positive benzidine test into a negative one; this point has not been investigated, but it is necessary to mention it because in arriving at the limits of sensitivity given above the vegetable intakes of the subjects whose faeces were examined were not controlled. Blood can be detected in simple aqueous solution by the benzidine test, when the oxyhaemoglobin has been diluted to between 0·15 and 0·20 mg. per 100 cc.

Lastly the limits of sensitivity of the different stages of Snapper's test [Snapper and Van Creveld, 1927] for blood were investigated. Faeces were employed which gave a negative benzidine test. 10 g. were thoroughly ground with 40 cc. of acetone, filtered, washed with acetone and sucked fairly dry. The precipitate was ground with 25 cc. of a mixture of glacial acetic acid, 1 part, and ethyl acetate, 2 parts and filtered. The filtrate was placed in one boiling-tube, and then first a solution of alkaline haematin and secondly a solution of alkaline porphyrin were placed in a second tube, the two tubes being

[1] If 0·85 % NaCl is used, owing to the salt content of the faeces (usually 2 to 3 g. per 100 g. fresh faeces) the mixture is hypertonic, and red cells are shrunken and less readily identified.

examined in line with the spectroscope exactly as described previously. It was found that the limits for detection of these two haemoglobin derivatives by this means were substantially the same as for aqueous solutions. Allowing for the necessary dilutions, and the haemoglobin content of the original blood, the results were as follows:

Minimum detected; mg. (as oxyhaemoglobin) per 100 g. fresh faeces.

Alkaline haematin	About 440
Alkaline porphyrin	„ 200

In the second part of the test 10 cc. of the filtered extract are treated with 2·5 cc. of pyridine and a few drops of yellow ammonium sulphide solution. This mixture was placed in one boiling-tube, and a solution of haemochromogen in the other. To another 10 cc. of the extract 5 cc. of 10 % HCl and 10 cc. of ether were added, mixed and allowed to separate. First the aqueous layer and then the ethereal layer were put in one boiling-tube, solutions of acid porphyrin and then of acid haematin respectively in the other tube. Again allowing for the dilutions *etc.*, the results were as follows:

Minimum detected; mg. (as oxyhaemoglobin) per 100 g. fresh faeces.

Haemochromogen...	About 65
Acid porphyrin	„ 140
Acid haematin	„ 200

Table II. *Limits of detection of blood pigments by various methods.*

Material tested	Oxyhaemoglobin or derivative	Bands	Technique	mg./100 cc.
Blood	Methaemoglobin	α	Spectroscopic	300
	Sulphaemoglobin	α	„	520
	Carboxyhaemoglobin	α and β	Proper technique, reversion spectroscope	1100
			Clinical technique, reversion spectroscope	7200
Urine	Haemoglobinuria	α and β	Spectroscopic	9·3
	Haematuria	α and β	„	11·5
		—	Centrifuge; red cells visible macroscopically	0·3
		—	Centrifuge; red cells visible under microscope	0·024
		—	Chemical { Phenolphthalin	3·5
			Chemical { Pyramidone	3·4
	Methaemoglobinuria	α and β and γ	Spectroscopic	110
	Porphyrinuria	α and β and γ (alkaline)	„	80
		β and γ (alkaline)	„	40
		α and β (acid)	Converted into acid porphyrin and concentrated 3 times	13
Faeces	Red blood cells	—	Microscopical	120
	Oxyhaemoglobin	α and β	Spectroscopic	360
	Haemoglobin and/or haematin	—	Benzidine (haemoglobin-free diet)	1·6
	Alkaline haematin	α	Snapper's test	440
	Alkaline porphyrin	α and β and γ		200
	Haemochromogen	α and β		65
	Acid porphyrin	α and β		140
	Acid haematin	α and γ		200

The haemochromogen and acid haematin portion of the test can be carried out directly on blood added to the faeces. This was done, and the same results were obtained.

The average results of the above tests on blood, urine and faeces are summarised in Table II.

Discussion.

A number of interesting points arise from the study of the relative values of clinical tests for blood when these are carried out approximately quantitatively. Attention will be drawn to a few to illustrate the value of the quantitative measurements, but obviously the data have a much wider application.

It is generally believed that by converting oxyhaemoglobin into haemochromogen, blood can be detected in much greater dilution [*cf.* Cole, 1928]. Actually the limiting concentration for haemochromogen is roughly half that for oxyhaemoglobin (limit for α band of haemochromogen 2·9, α band of oxyhaemoglobin 5·0 mg. per 100 cc.). This is of practical importance because, if in the process of converting oxyhaemoglobin into haemochromogen the dilution is more than twofold, the test is made less sensitive.

This is but a single instance of the general value of knowing the relative intensities of the absorption bands of haemoglobin and its derivatives. They can be calculated from the data in Table I. The results (to the nearest whole number) are shown in Table III, the intensity of the α band of oxyhaemoglobin being taken as 100.

Table III. *Relative intensities (approximate) of absorption bands of haemoglobin derivatives (α band of oxyhaemoglobin = 100).*

Absorption band	α	β	γ	δ
Oxyhaemoglobin	100	57	—	—
Carboxyhaemoglobin	63	45	—	—
Sulphaemoglobin	10	35	16	—
Reduced haemoglobin	10	—	—	—
Methaemoglobin	9	5	7	?
Acid haematin	7	2	6	?
Alkaline haematin	3	—	—	—
Haemochromogen	173	25	—	—
Acid porphyrin	14	47	—	—
Alkaline porphyrin	7	13	20	?

The colours of the haemoglobin derivatives when diluted to the spectroscopic limit vary not only in tint but also in intensity (see Table I). This again is of practical importance. Thus if a sample of urine (1″ thick or less) is red, and bands of oxyhaemoglobin are not visible, the red colour cannot be due to oxyhaemoglobin. On the other hand urine may be quite definitely yellow-brown due to methaemoglobin, and yet the absorption bands of methaemoglobin may not be visible when a sample 1″ thick is examined with the spectroscope. Similarly acid haematin may be present in a solution in sufficient concentration to make the colour a well-marked brown, and yet when a column 1″ thick is viewed with the spectroscope no bands are seen, though a layer several inches thick may show the bands. To overcome these difficulties it is a common practice clinically to examine the urine or other fluid in a conical receiver, moving the spectroscope upwards and downwards to view layers of different thicknesses (the diameter of the usual type of conical receiver varies from about $\frac{1}{2}$″ at the bottom to about 3″ at the top). From the data presented in the table it is easy to calculate the spectroscopic limit for other thicknesses of fluid.

Not only methaemoglobin and acid haematin, but also alkaline haematin and alkaline porphyrin, should be considered when examining brown solutions with a spectroscope; it will be seen from Table I that the spectroscopic test is relatively insensitive for all four. Furthermore it should be remembered that the porphyrins, being iron-free, do not give positive reactions with the catalytic group of chemical tests (benzidine, phenolphthalin, *etc.*).

Methaemoglobin can be detected in blood, using the technique described, when about 0·3 g., and sulphaemoglobin when about 0·52 g., are present per 100 cc. If, and only if, the total haemoglobin is of the order usually found in health, some 14 g. per 100 cc., methaemoglobin is detectable when it accounts for about 2·1 %, and sulphaemoglobin when it accounts for about 3·7 %, of the total haemoglobin. This second method of expressing results however may be misleading. Thus, for instance, methaemoglobin amounting to 2·1 % of a total of 14 g. haemoglobin would be detectable by the spectroscopic technique outlined, whereas methaemoglobin amounting to 2·1 % of a total 10 g. haemoglobin would not. Furthermore it is the number of grams of active oxyhaemoglobin per 100 cc. which is important for purposes of respiration; a statement of the percentage of the total haemoglobin which is in the form of oxyhaemoglobin is of very limited value unless the total haemoglobin is known. Whichever method is used for expressing the spectroscopic limit for methaemoglobin or sulphaemoglobin, it is valueless unless accompanied by a knowledge of the thickness of the solution examined. Even the statement, which has appeared in more than one publication, that the α band of sulphaemoglobin can be detected spectroscopically when sulphaemoglobin accounts for 10 % of the total haemoglobin might be inaccurate and certainly requires proper qualification.

For practical purposes the simplest test for haemoglobinuria is the spectroscopic examination of the urine from which any cells have been removed by centrifuging. The catalytic chemical methods, applied to the cleared urine, are more sensitive, and by suitable alterations in technique they can easily be made still more sensitive than those applied in this investigation [*cf.* Ingham, 1932]. At the same time these chemical methods are not specific; enzymes and iodides may give false positives, and their exclusion complicates the tests. Moreover, the spectroscopic method can easily be made a little more sensitive by examining a thicker layer. If no absorption bands are seen when a layer of the cleared urine 2″ or 3″ thick is examined spectroscopically, no significant degree of haemoglobinuria is present, and ultra-sensitive chemical tests are superfluous.

Similarly in haematuria the microscopical examination of the centrifuged deposit for red cells is by far the simplest test; and moreover it is also the most sensitive. The technique of Ingham [1932] is probably the most delicate of all the chemical methods. He claims that his hyperol-benzidine reaction will detect 1 cc. of blood in from 200,000 to 260,000 cc. of urine. Assuming that he implies normal blood, for which a reasonable average haemoglobin content is 14 g. per 100 cc., his results are equivalent to 0·070 to 0·054 mg. oxyhaemoglobin per 100 cc. urine. As shown above the microscope will readily detect red cells of an amount corresponding to 0·024 mg. oxyhaemoglobin per 100 cc., and with a little experience the sensitivity of the microscopical examination becomes even greater.

It is not a common practice to examine diluted and cleared aqueous faecal extracts spectroscopically for oxyhaemoglobin, but such a test would be useful, as a check for instance on Snapper's test. Thus by comparing the oxyhaemoglobin content of the faeces with the acid haematin content as determined by Snapper's method, it is possible to judge approximately how much of the acid

haematin was formed from oxyhaemoglobin during the test, and how much from alkaline haematin passed as such in the faeces.

Finally this illustrates the possibility of computing approximately the content of oxyhaemoglobin or of a derivative thereof in blood, urine or faeces. For example the amount of blood in urine can be calculated roughly from the spectroscopic limit for oxyhaemoglobin in water (or urine), and the number of times that urine must be diluted with water (or normal urine) to reach the spectroscopic limit. Even in the case of blood it is surprising how closely the spectroscopic results approximate to the haemoglobin content determined from the oxygen-combining power:

Oxyhaemoglobin (g. per 100 cc.) calculated from average spectroscopic limit for α band (5 mg. per 100 cc.) and dilution factor	Oxyhaemoglobin (g. per 100 cc.) from oxygen-combining power
14·0	13·7
12·5	12·5
20·0	18·9
15·0	15·4
12·5	12·7

SUMMARY.

1. The concentrations of oxyhaemoglobin and its derivatives which can just be detected by a given spectroscopic technique have been determined. The relative value of spectroscopic tests for the different derivatives has thus been put on a quantitative basis.

2. The sensitivities of spectroscopic tests for those haemoglobin derivatives which are of clinical importance in examining blood, urine and faeces have similarly been defined, making due allowance for variations in technique necessitated by the nature of blood and the excretions.

3. These results have been compared with the sensitivity determined for certain of the chemical catalytic reactions and for the microscopical detection of red blood corpuscles.

4. The practical value of some of the above data in clinical investigations is discussed.

I am much indebted to Dr G. A. Harrison for suggesting this investigation, and for his help in compiling the paper.

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XIX. THE PRODUCTION OF VITAMIN D BY IRRADIATION OF ERGOSTEROL THROUGH THE EPIDERMIS OF A RAT.

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(*Received December 15th, 1932.*)

In an earlier publication concerning the permeability of human epidermis to ultra-violet radiations [1931] the suggestion was put forward that enough ultra-violet rays of sufficiently short wave-length to activate ergosterol might penetrate the epidermis and reach the ergosterol contained in the capillaries lying just below it. Experiments were described which indicated that the percentage of radiations transmitted varied from 44 % for a wave-length of 300 $m\mu$ to 2·9 % for wave-length 289 $m\mu$.

Proof, however, was still wanting that this percentage of transmitted light was sufficient to produce vitamin D in the manner suggested. To test the matter an experiment was devised in which the criterion for the formation of vitamin D was the measurement of the characteristic change produced by irradiation in the ultra-violet absorption spectrum of ergosterol, as described by Bourdillon *et al.* [1929]. A biological test was also made.

Method of spectrographic investigation.

The solution of ergosterol was held in a quartz tube $3\frac{1}{4}$ inches long with an internal diameter of $\frac{1}{2}$ an inch. Near the centre a constriction was made in the bore, producing a flat-walled portion of the tube $\frac{1}{2}$ an inch long with an internal cross section of $\frac{3}{32}$ of an inch. An alcoholic solution of ergosterol (0·003 % in absolute alcohol) was then poured into the tube to the level of $\frac{5}{8}$ of an inch from the top, and it was closed with an india-rubber stopper, the joint being sealed with collodion. Through the stopper two narrow glass tubes were passed far enough to project rather less than $\frac{1}{8}$ of an inch into the quartz tube. Through one of these nitrogen previously washed in sodium pyrogallate was passed to displace the air left in the tube. While the nitrogen was still passing, the narrow tubes were sealed off with a jeweller's blowpipe, leaving the ergosterol solution sealed in the tube, with a space filled with nitrogen above it. The quartz tube was then inserted into a wooden tube, which had been bored to fit it exactly and soaked in molten paraffin wax to prevent swelling of the wood during subsequent exposure to moisture. In the centre of the wooden case a window was cut $\frac{3}{8}$ of an inch wide running half way round. The quartz tube fitted too well to rotate inside the case and was held in longitudinal position by pads of cotton wool and corks at each end.

The wooden case was attached by screws along the diameter of a perpendicular wooden pulley on a bicycle hub. The pulley was rotated slowly by means of an electrically driven series of belt gears, whereby the wooden case and the quartz tube were also rotated in the plane of their length.

The nitrogen left in the tube was thus caused to travel continuously from one end of the tube to the other, and the requisite stirring of the ergosterol was produced.

A drip feed of tap-water was adjusted to fall on a specially prepared piece of rat epidermis covering the outer side of the flat quartz tube, thus keeping it moist. The window was fixed exactly over the centre of the pulley so that it remained in constant position below the drip.

The epidermis used in the experiment was that of a 24-hour old rat, which was chosen as being more conveniently obtainable than that of a human being. The epidermis was detached from the dermis by rolling a test tube of almost boiling water along the back and sides of a rat which had previously been anaesthetised with ether. After a few minutes, the epidermis became detached from the dermis, but it was not necessary to wait until a true blister was formed. A very shallow incision in the posterior end of the scald made possible the insertion of a blunt pointed ligature needle. By this means the epidermis was raised all round the scalded area, cut off with fine scissors and placed in tap-water. The rat was then destroyed by decapitation.

A section of the blister examined under the microscope showed that separation had taken place at such depth as to leave a layer of connective tissue, only two cells thick, superficial to the capillaries. Irradiation therefore took place through practically the entire thickness of epidermis superficial to the capillaries.

The piece of epidermis was spread over the flat area of the quartz tube, beyond the edges of which it extended. It was held in position by collodion applied round the margin. This was done before the tube was put in the wooden case. Owing to the window in the wooden case being smaller than the area covered by epidermis, it did not appear possible for any light to penetrate the quartz tube except after passage through the epidermis.

The irradiation was carried out by means of a quartz mercury vapour arc consuming 4 ampères and placed at 10 inches from the epidermis. A large flat quartz cell, the internal cross section of which was 1 cm. filled with 1 % xylene in absolute alcohol was placed in a line with the lamp and 7 inches from it. The purpose of this cell was to filter off all radiation shorter than a wave-length of 286 $m\mu$, thereby preventing the destruction of vitamin D by radiations of shorter wave-lengths.

Since there were no definite data on which to base the length of exposure required, one of 10 hours' duration was used. A concentration of 0·003 % ergosterol in absolute alcohol was chosen because the peak of the absorption band at 280 $m\mu$ of such a solution was situated at a point two thirds of the way through the total range of exposures allowed by a Hilger quartz spectrograph, model E.

Any rise in the height of this peak due to the production of vitamin D could be shown on a similar plate without dilution being required, whereby comparison of the two spectrograms would be much simplified.

The 10-hour period of irradiation was divided into two portions, 6 hours on the first, and 4 hours on the second day. The wooden case, with the quartz tube and epidermis left *in situ*, was unscrewed from the pulley and during the interval between the two exposures immersed in a vessel of tap-water at just above 0°.

At the end of the 10 hours some of the solution was transferred to the tube of a Hilger sector photometer and photographed. The resulting spectrogram was compared with that of the original solution before irradiation.

Discussion of result.

In Fig. 1 the absorption curves have been set out. Owing to the fact that the spectrogram of the irradiated solution is that of a mixture of which the concentration of the constituents is unknown, it was not thought advisable to attempt to represent the ordinates as coefficients of absorption, and they have therefore been left as logarithms of the ratio of the incident to the transmitted light (log I/I').

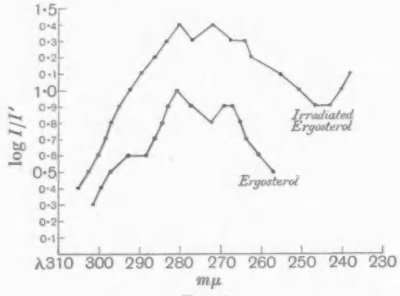


Fig. 1.

The curves show that the irradiated solution has a higher absorption in the region of 280 $m\mu$ than that of the unirradiated. This is in keeping with the findings of Bourdillon *et al.* [1929], and is therefore strong evidence of the formation of vitamin D from ergosterol.

Biological test.

In order to confirm, or otherwise, the result thus obtained by the spectrographic technique, it was decided to test a solution of ergosterol, irradiated through epidermis in the same way, biologically for vitamin D. The method used was the curative one, rickets being assessed by radiography according to the method of Bourdillon *et al.* [1931].

The irradiation was repeated except that the concentration of the ergosterol solution was increased from 0·003 % to 0·01 % since it was desired to obtain a large enough supply of the test material to feed the necessary number of animals. The only advantage of the low concentration used in the previous experiment was its suitability for spectrographic examination.

Nine rats were fed on a rachitogenic diet for 21 days and were found by radiography to be suitably rachitic at the end of that period.

Three of the rats then received for 14 days daily doses of calciferol of the value of 4, 2 and 1 units of vitamin D respectively.

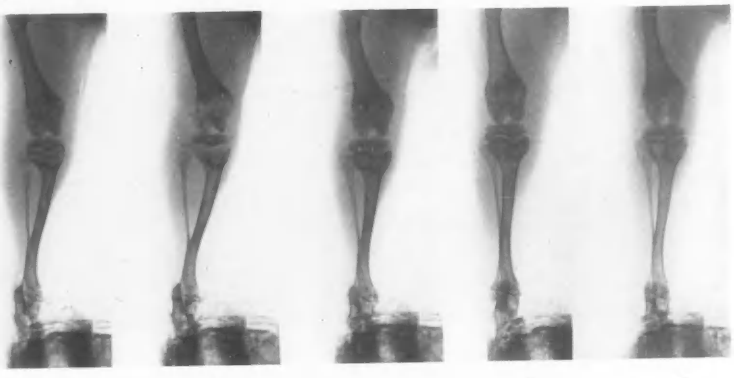
The other six received for the same period the solution of ergosterol irradiated through epidermis in doses proportionate to 1/250, 1/1000 and 1/2500 mg. of the original ergosterol, two animals receiving each dose.

After 14 days the rats were killed and again radiographed. The radiograms are reproduced in Plate I.

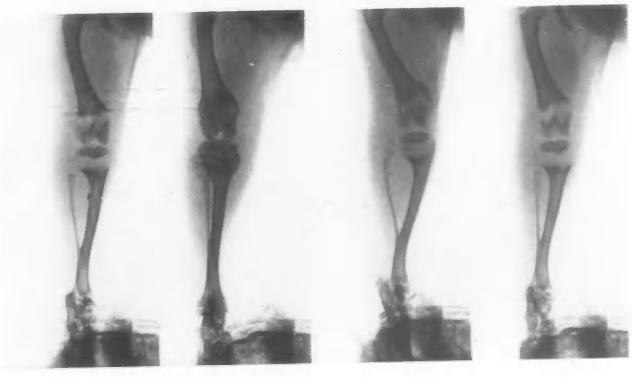
Numbers 20, 22 and 24 received the three doses of calciferol and show proportionate degrees of good healing.

Numbers 21 and 27 received the dose of 1/250 mg. as described above and show good healing.

Numbers 23 and 26 received 1/1000 mg. of the same and show some healing, while numbers 25 and 28 received 1/2500 mg. and show none.



24 23 22 21 20



28 27 26 25

Discussion.

From examination of the radiograms, Plate I, it is seen that the calcification produced by daily doses of the solution under test, proportionate to 1/250 mg. of the original ergosterol, is equal to that produced by the administration of 2 units of vitamin D as calciferol. It is therefore possible to say that in the present conditions of irradiation through epidermis, 1/250 mg. of ergosterol yielded 2 units of vitamin D, and 1·0 mg. would yield 500.

Calciferol has a value of 40,000 units of vitamin D per mg., so that 1·0 mg. of ergosterol after irradiation through rat epidermis in the way specified, possessed $\frac{1}{80}$th of the antirachitic potency of pure calciferol.

Summary.

1. Ergosterol, in alcoholic solution with exclusion of oxygen, was irradiated with ultra-violet radiation through the epidermis of a day-old rat, by means of a specially devised apparatus.

2. Examined spectrographically, the resulting solution showed the changes in the ultra-violet absorption spectrum which are characteristic of the formation of vitamin D.

3. Examined biologically for the presence of vitamin D, the solution after irradiation was found to have developed an antirachitic potency, corresponding with 500 international units of vitamin D per mg. of the original ergosterol. Pure calciferol contains 40,000 such units per mg.

My thanks are due to the Lister Institute of Preventive Medicine for facilities in carrying out this experiment, and to Dr Webster, Miss Bruce and Mr Philpot for doing the biological test. I am also grateful to the Medical Research Council for the loan of the large quartz cell.

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XX. SOME OBSERVATIONS ON THE RESPIRATORY CATALYSTS PRESENT IN THE SPORES AND VEGETATIVE CELLS OF CERTAIN AEROBIC BACILLI.

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(*Received December 15th, 1932.*)

RUEHLE [1923] studied spores obtained from twelve unidentified species of aerobic bacilli. No reduction of methylene blue by suspensions of these spores was found even after 5 hours at 45–48°, but no oxidisable substrate was added. He states that he obtained a slight positive indophenol reaction with such suspensions and a strong catalase reaction, the last-named test being positive even after boiling the spores. Cook [1931] prepared spore suspensions from a strain of *Bacillus subtilis* obtained from the National Collection of Type Cultures. Such suspensions reduced methylene blue at 40° in the presence of glucose even after heating to a temperature somewhat lower than that of boiling water. Spores exposed to the temperature of boiling water for a fairly long time no longer reduced methylene blue under these conditions. Suspensions of vegetative cells of the same organism did not reduce methylene blue at 40° in the presence of glucose. Glucose was oxidised aerobically by both spores and vegetative cells, and the data given suggest that spores are as active in this respect as the vegetative cells which produce them.

EXPERIMENTAL.

The following cultures have been employed:

B. subtilis I. Strain employed by Cook [1931].
B. subtilis II. Strain employed by Harrison, Tarr and Hibbert [1930].
B. mesentericus. Strain employed by Harrison, Tarr and Hibbert [1930].
B. megatherium. National Collection of Type Cultures, No. 654.

In a previous paper [Tarr, 1932] it was shown that endospore formation takes place in almost every cell in a given culture when aerobic sporing bacilli are cultivated under strictly aerobic conditions on the surface of agar media which contain only a relatively small amount of available nutrient material. This result has rendered the preparation of spore suspensions practically free from vegetative cells a comparatively simple matter. The medium for the production of spores had the following composition: Witte peptone, 5 g.; Liebig's beef extract, 2 g.; NaCl, 5 g.; agar, 30 g. and water to 1 litre. The medium employed for the preparation of vegetative cells contained 10 g. of Liebig's beef extract in place of 2 g. Both media were adjusted colorimetrically to p_H 7·4 and sterilised in 1 litre Roux flasks for 20 minutes at 15 lb. steam pressure. The surface of the

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solidified medium was inoculated from a light suspension of the organism being studied. For the preparation of vegetative cells the flasks were incubated for 12 hours at 37°, while an incubation period of ten days at the same temperature was allowed in the case of spores. Both spores and vegetative cells were washed twice on the centrifuge with Ringer's solution and were then suspended in the same liquid to give a suitable concentration of cells for experimental work. The nitrogen percentage of the dry weight was determined on material washed free from inorganic salts and dried to constant weight at 100°.

	Nitrogen % of dry weight
B. subtilis I vegetative cells	10·64
B. subtilis I spores	10·43
B. mesentericus spores	10·20
B. megatherium spores	10·36
B. subtilis II spores	9·37

The values given in the following series of experiments for the dry weight employed were obtained by determining the amount of nitrogen in a 1 cc. sample and then multiplying by the above factors. All spore suspensions were stored at 0–3° and were used within 10 days of preparation, while vegetative cells were kept at room temperature and were used within 12 hours of preparation.

The dehydrogenase activity was studied in Thunberg tubes containing 1 cc. of the suspension, 1 cc. of $M/5$ phosphate buffer p_H 7·3, 1 cc. of distilled water, 1 cc. of the substrate solution and 1 cc. of a 1/5000 solution of methylene blue. The endogenous reduction of methylene blue was studied by substituting 1 cc. of water for the substrate solution. In a large number of the experiments in which the dehydrogenase activity of spores was studied the cells were incubated for 30 minutes at 40° in a thermostat in the presence of the buffer solution and the substrate, the methylene blue and solutions of inhibiting substances being added immediately prior to evacuation. All tubes were evacuated for 1½ minutes and immediately placed in a water thermostat at 40°, and the time required for 90 % reduction of methylene blue was recorded to the nearest half minute. The effects of 5 % ethylurethane and a saturated solution of phenylurethane were studied by adding 0·25 g. of the former and 5 mg. of the latter to the solutions in the Thunberg tubes and mixing thoroughly before evacuation. The action of KCN was investigated by placing 0·5 cc. of a solution of the appropriate concentration in the curved end of the hollow stopper of a specially constructed Thunberg tube [Keilin, 1929], the fluids in the stopper and tube being mixed immediately subsequent to evacuation and prior to placing in the thermostat. Experience showed that $M/1000$ and $M/500$ KCN (final concentrations) caused no significant alteration in the p_H of the solutions, while $M/250$ caused a change from 7·3 to about 7·6.

Oxygen uptakes were studied in Barcroft differential manometers, at 40° unless otherwise stated. In each experiment the right-hand cup contained 1 cc. of suspension, 1 cc. of $M/5$ phosphate buffer p_H 7·3 (unless otherwise stated), 0·5 cc. of $M/10$ glucose solution and sufficient distilled water to bring the final volume of liquid in the flask to 3 cc., thus allowing for the addition of solutions of such inhibiting substances as KCN and H_2S. The left-hand cup contained the same constituents with the exception of the glucose solution which was replaced by water. In experiments in which the endogenous oxygen uptake was investigated the right-hand cup contained 1 cc. of suspension, 1 cc. of $M/5$ phosphate buffer p_H 7·3 and 1 cc. of water, while the left-hand cup contained 2 cc. of water and 1 cc. of the buffer solution. In order to equalise conditions as much as

possible the inhibiting substances were always added to the solutions both in the right- and left-hand cups of the manometer. Small rolls of filter-paper moistened with 6 % KOH as suggested by Keilin [see Dixon and Elliott, 1930] were used to absorb any CO_2 that might be produced.

The H_2S employed was prepared by bringing dilute solutions of pure Na_2S to p_H 7·6–7·8 with dilute HCl; 0·3 cc. of such a solution of the appropriate concentration being added to the solution in the manometer cup immediately before commencing the experiment. The effect due to H_2S usually disappeared after the first 30 minutes of the experiment, because, even though phosphate buffer of p_H 7·8 was employed in such experiments, the reagent rapidly distilled over to the centre tube containing the KOH. The effect of cyanide was determined by adding 0·3 cc. of a solution of pure KCN of the appropriate concentration to the liquid in the manometer cup by means of a small tube [Keilin, 1929] as soon as the velocity of the oxidation of glucose became constant. 5 % ethylurethane and saturated phenylurethane were investigated by placing 0·15 g. of the former and 5 mg. of the latter in each manometer cup before adding the experimental solutions. The manometers were equilibrated for 5–7 minutes before commencing the experiments. Duplicate determinations agreeing within 5 % were considered sufficiently accurate.

The dehydrogenase activity of spores and vegetative cells.

Table I shows the effects of different treatments on the activity of the glucose dehydrogenase of *B. subtilis* I spores.

Table I. *The influence of previous treatment on the activity of the glucose dehydrogenase of spores of* B. subtilis *I.*

4·7 mg. dry weight in each exp. No sign of growth was ever observed in anaerobic experiments, and results recorded later show that in no case can the activity due to spores be accounted for by vegetative cells.

Treatment	Reduction time in min. at 40°
(1) Freshly prepared spores	60
(2) Spores directly after heating to 80° for 30 mins.	40
(3) Freshly prepared spores after incubating 30 mins. at 40° in the presence of glucose and buffer prior to studying reduction	40
(4) Freshly prepared spores heated to 80° for 30 mins. and then treated as (3)	12
(5) Freshly prepared spores heated as in (2) and incubated for 30 mins. in buffer solution at 40° prior to studying reduction	30

It is evident from Table I that spore suspensions previously heated to 80° for 30 minutes dehydrogenate glucose more rapidly than do freshly prepared unheated spores, and also that both heated and unheated spores dehydrogenate glucose much more rapidly if incubated aerobically for 30 minutes at 40° in the presence of their substrate. That the presence of glucose stimulates the formation of a glucose dehydrogenase is shown by the fact that spores incubated in the absence of glucose do not dehydrogenate glucose nearly as rapidly as spores incubated in the presence of this sugar.

In Table II a comparison of the dehydrogenase activities of the spores of four different strains is given. Except in the case of the controls the spores were all heated to 80° for 30 minutes and then incubated for 30 minutes at 40° in the presence of the substrate and buffer. In the case of the controls the spores were

Table II. *Dehydrogenase activity of the spores of four species of aerobic bacilli.*

B. subtilis I 4·7 mg. dry wt. per exp.
B. subtilis II 4·2 ,, ,,
B. mesentericus 4·4 ,, ,,
B. megatherium 6·3 ,, ,,

Donator	Reduction time of *M*/30,000 methylene blue in minutes			
	B. subtilis I	*B. subtilis* II	*B. mesentericus*	*B. megatherium*
None (control)	*	*	*	60
M/250 lactate	240	*	*	49
M/100 glucose	12	23	25	4
,, galactose	15	28	36	8
,, fructose	19	22	42	8
,, mannose	20	29	43	14
,, sucrose	13½	39	31	5
,, lactose	70	90	120	41
,, maltose	17	25	37	11

* Not reduced within 24 hours.

heated but incubated in buffer solution alone. Although spores are not quite as active as vegetative cells (compare with Table III), they can dehydrogenate hexoses and disaccharides fairly actively, the activity varying a great deal with the species under investigation. *M*/250 formate, acetate, oxalate, fumarate and succinate, and *M*/100 glycerol, mannitol, arabinose and xylose were not activated within 16 hours.

Table III. *The effect of KCN, 5 % ethylurethane and saturated phenylurethane on the glucose dehydrogenase of the spores and vegetative cells of* B. subtilis *I.*

Vegetative cells 5·2 mg. dry wt. per exp.
Spores 9·4 ,, ,,

Type of experiment	Reduction time of *M*/30,000 methylene blue in minutes in the case of	
	Vegetative cells	Spores
Endogenous reduction	6·0	—
M/60 glucose	3·5	6
,, in *M*/250 KCN	6·0	6
,, in *M*/500 KCN	4·5	6
,, in *M*/1000 KCN	4·5	6
,, + sat. phenylurethane	3·5	6
,, + 5 % ethylurethane	*	8

* Not reduced in 16 hours.

The effect of cyanide, ethylurethane and phenylurethane on the activity of the glucose dehydrogenase activity of the spores and vegetative cells of the same strain of *B. subtilis* is shown in Table III. The spores were heated to 80° for 30 minutes and then incubated for 30 minutes at 40° in the presence of their substrates before the tubes were evacuated, the methylene blue being added immediately prior to evacuation. Cyanide causes some inhibition in the velocity of dehydrogenation of glucose in the case of vegetative cells, but is without influence on the same process in the case of spores. Phenylurethane causes no retardation in the reaction with either vegetative cells or spores, while ethylurethane entirely inhibits the former but not the latter. However, additional

experiments have shown that, if spores are merely heated to 80° for 30 minutes and the dehydrogenation of glucose immediately studied without previous incubation for 30 minutes in the presence of the substrate, the reaction is entirely inhibited by ethylurethane, but not by phenylurethane or cyanide. No explanation can at present be offered for this curious difference in the action of ethylurethane.

Aerobic oxidations.

In Table IV the abilities of vegetative cells and spores of four species to oxidise glucose and their own cell material (endogenous oxidation) are compared. The relative oxygen uptakes are shown to be much greater in the case of vegetative cells than in spores of the same species. The endogenous uptake in the

Table IV. *A comparison of the endogenous oxygen uptakes and oxygen uptakes in M/60 glucose by vegetative cells and heated spores.*

Species	Oxygen uptake in mm.³ per g. of dry wt. per hour	
	Endogenous	In *M*/60 glucose
B. subtilis I vegetative cells	—	88,000
B. subtilis I spores	2,000	31,000
B. subtilis II ,,	600	27,000
B. megatherium ,,	4,000	43,000
B. mesentericus ,,	500	20,000

case of spores is only a small fraction of that obtained in glucose, and considerable variation in activity exists in the case of spores of different species. The spores in the above experiments were heated to 80° for 30 minutes immediately

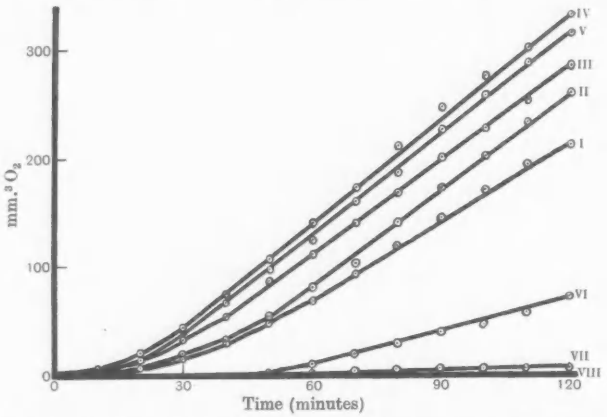

Fig. 1. Oxygen uptakes in glucose at 40° by spores of *B. subtilis* I after previous heating to the temperatures given. 9·5 mg. dry wt. per exp. I. Unheated control; II. Heated to 60° for 30 minutes; III. Heated to 70° for 30 minutes; IV. Heated to 80° for 30 minutes; V. Heated to 90° for 30 minutes; VI. Heated to 99·5° for 5 minutes; VII. Heated to 99·5° for 30 minutes. VIII. Autoclaved (15 lb. for 20 minutes).

prior to use, and the oxygen uptakes calculated over a 1 hour period after the velocity of oxidation became constant (see Fig. 1). These results indicate that some active respiratory catalyst exists in the spores of aerobic bacilli.

The effect of heating spore suspensions of *B. subtilis* I prior to studying the oxidation of glucose is shown in Fig. 1. Heating to temperatures between 60° and 90° for 30 minutes occasions marked increases in the oxygen consumption during the first 40–60 minutes of the experiment, the most effective temperature being 80°. At 90° the increase is not as great as at 80°, at 99·5° for 5–30 minutes the catalyst is largely inactivated, and autoclaving, which kills the spores, destroys the active catalyst completely. Fig. 1 clearly shows that the real effect caused by previous heating to temperatures between 60° and 90° for 30 minutes is to establish a constant velocity of oxidation earlier than in unheated spores. Thus in unheated spores the oxidation velocity does not become constant until about 50–60 minutes after commencing the experiment, while previous heating to 80° or 90° for 30 minutes causes the velocity of oxidation to become constant some 30 minutes after commencing the experiment. It is interesting to note that the final constant velocity of oxidation attained by heating spores is only slightly greater than that given by unheated spores. All spore suspensions employed in further experiments were heated to 80° for 30 minutes immediately prior to use.

It is interesting to note that "plate" counts, using Hartley's beef digest agar as substrate, showed, in a representative experiment, 29×10^9 colonies per cc. of suspension before heating to 80° for 30 minutes, and 25×10^9 subsequent to heating. This shows that a relatively small proportion of spores is actually killed by such treatment.

Fig. 2 shows the p_H-activity curves of the system oxidising glucose in *B. subtilis* I spores. The oxidation in each case was studied over a 1 hour period as soon as the velocity of oxidation became constant. Phthalate-NaOH; KH_2PO_4-NaOH and borate-NaOH buffers (Clark and Lubs) were employed at the rate of 1 cc. of $M/5$ solution per cup, the p_H of the solutions in the manometer cups being checked by quinhydrone or hydrogen electrodes at the commencement and conclusion of the experiments. In Fig. 2 curves are plotted for the p_H values obtained at the commencement and conclusion of each experiment, and the true p_H-activity curve must lie somewhere between them. The general shape of the curve is similar to that obtained by Cook and Alcock [1931] for the oxidation of lactate by *B. coli*. Phthalate buffer only slightly retards the velocity of oxidation, while borate inhibits the oxidation markedly. The optimum p_H is about 6·5, with a very sharp decline in activity between p_H 6 and 5, while in the alkaline range from p_H 7 to 8·5 the decline in activity is slight. Beyond p_H 8·5 the decline in activity is extremely rapid.

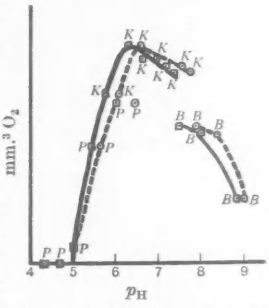

Fig. 2. p_H-activity curves for glucose oxidation at 40° by heated spores of *B. subtilis* I. 4·9 mg. dry wt. per exp.

Initial p_H curve.
Final p_H curve.
P = phthalate-NaOH buffer.
K = KH_2PO_4-NaOH buffer.
B = Borate-NaOH.

The effect of temperature on the velocity of glucose oxidation by heated spores and vegetative cells of *B. subtilis* I is shown in Figs. 3 and 4 respectively. In the case of vegetative cells the velocity of oxidation was studied over the first hour of the experiment, while in the case of heated spores the oxygen uptakes during the second hour of the experiment were used to indicate the oxidation velocity. The general shape of the curves in each case is closely similar, the optimum temperature being about 50° with a very rapid decline in activity at about 55°. However, vegetative cells were found to oxidise glucose at tempera-

tures as low as 15–20°, while in the case of spores no oxygen uptake was registered at 20°. The fact that spores possess a respiratory catalyst which will withstand heating to 80° for 30 minutes and which, when active, is sensitive to temperatures above 50°, is to be emphasised. Absolutely no motile vegetative cells were observed in spore preparations which had oxidised glucose for 2 hours at 50° and

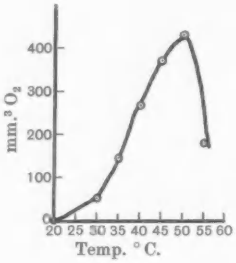

Fig. 3.

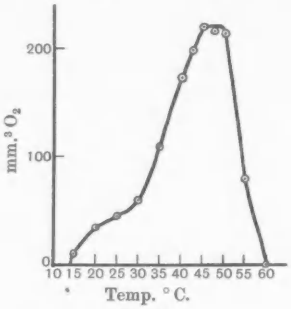

Fig. 4.

Fig. 3. Effect of temperature on the velocity of glucose oxidation by heated spores of *B. subtilis* I. 9·25 mg. dry wt. per exp.

Fig. 4. Effect of temperature on the velocity of glucose oxidation by vegetative cells of *B. subtilis* I. 5·2 mg. dry wt. per exp.

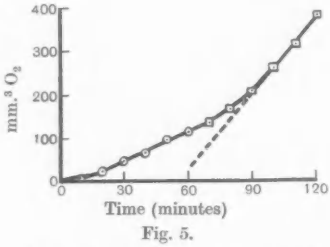

Fig. 5.

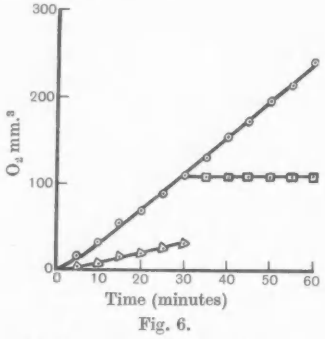

Fig. 6.

Fig. 5. The effect of cooling to 50° on oxidation of glucose at 55°, by heated spores of *B. subtilis* I. 9·25 mg. dry wt. per exp.

⊙—⊙—⊙ Oxidation at 55°.
⊡—⊡—⊡ ,, ,, 50°.

Fig. 6. Effect of *M*/500 H_2S and KCN on glucose oxidation at 40° by vegetative cells of *B. subtilis* I. 2·8 mg. dry wt. per exp.

⊙—⊙—⊙ Control.
⊡—⊡—⊡ *M*/500 KCN added.
△—△—△ *M*/500 H_2S added.

this shows that such oxygen uptakes are not due to vegetative cells. At 40° a few motile vegetative cells were usually observed after the second hour of such experiments, but not after the first hour. Further experiments show that the respiration occurring in spores is of a different type from that occurring in vegetative cells.

In order to ascertain whether or not the decrease in the velocity of oxidation of glucose at 55° by vegetative cells and heated spores of *B. subtilis* I was reversible or not, two experiments were made. In the first heated spores were allowed to oxidise glucose for 1 hour at 55°, and at the conclusion of this time the thermostat was cooled within 5 minutes to 50°, the manometers being equilibrated with the taps open during the cooling process, and readings were taken for 1 hour at 50°. The results which are shown in Fig. 5 indicate that there is a lag period, and that then, about 10 minutes after cooling, there is a gradual increase in the oxidation velocity until, during the final 30 minutes of the experiment, the velocity becomes approximately constant. Calculation showed that the rate of oxidation of glucose subsequent to cooling was about 160 % greater than before the temperature was lowered. Thus with spores the partial inactivation which takes place at 55° is reversible. A similar experiment with vegetative cells proved that, in this case, the inactivation at 55° is entirely irreversible, the velocity after cooling being even lower than that recorded prior to lowering the temperature.

The effects of KCN and H_2S on the oxidation of glucose by both spores and vegetative cells of *B. subtilis* I were next investigated. Fig. 6 shows that $M/500$ KCN inhibits glucose oxidation by vegetative cells of this organism entirely. The action of $M/500$ H_2S was not so pronounced, the inhibition calculated for the first 30 minutes of the experiment being 70 %. Naturally the H_2S concentration falls off extremely rapidly owing to its volatilisation to the centre tube containing the KOH, and the inhibition would probably be greater if the H_2S concentration remained constant. The effect of different concentrations of KCN on the oxidation of glucose by spores is shown in Fig. 7. It will be seen that spores are not

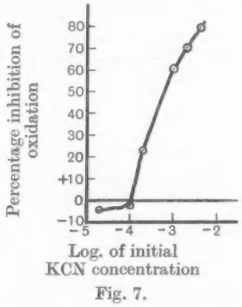

Fig. 7.

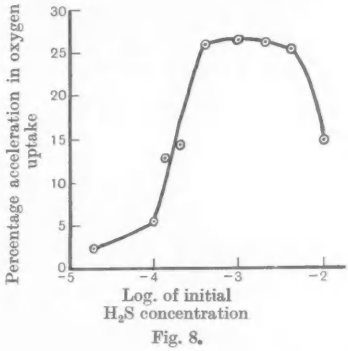

Fig. 8.

Fig. 7. Effect of KCN on the velocity of glucose oxidation at 40° by heated spores of *B. subtilis* I. 9·4 mg. dry wt. per exp.

Fig. 8. Effect of various H_2S concentration on oxidation of glucose at 40° by heated *B. subtilis* I spores. 9·1 mg. dry wt. per exp.

as sensitive to cyanide as are vegetative cells, $M/500$ KCN causing only 70 % inhibition in glucose oxidation by the former, and complete inhibition in the case of the latter. Both $M/10{,}000$ and $M/50{,}000$ KCN caused a slight acceleration in the velocity of glucose oxidation by spores. The effect of sulphide on the rate of the oxidation of glucose by spores is very interesting as will be seen from Fig. 8, for, in contrast to its effect on this process in vegetative cells, it actually

accelerates the reaction. In concentrations of $M/2500$ and $M/250$ H_2S during the first 30 minutes of the experiment the acceleration is about the same, namely 25–27 %, and at both higher and lower concentrations the percentage of acceleration is lower. It will be seen later that, in the case of spores of the other three species studied, H_2S caused no acceleration of the oxygen uptake, and apparently had no influence whatever on glucose oxidation.

Experiments showed that 5 % ethylurethane and saturated phenylurethane both inhibited the oxidation of glucose by the spores of *B. subtilis* I completely. Glucose oxidation by vegetative cells of the same bacillus was entirely inhibited by 5 % ethylurethane, while a saturated solution of phenylurethane decreased the velocity of the reaction some 75 % during the first 30 minutes of the experiment, and caused almost complete inhibition during the last 30 minutes.

Table V. *The effects of cyanide and sulphide on glucose oxidation by spores and vegetative cells.*

Experiment	Effect of $M/500$ H_2S	Effect of $M/500$ KCN
B. subtilis I vegetative cells	70 % inhibition	100 % inhibition
B. subtilis I spores	27 % acceleration	71 % ,,
B. subtilis II ,,	No effect	72 % ,,
B. mesentericus ,,	,,	67 % ,,
B. megatherium ,,	,,	48 % ,,

Table V summarises the effects of $M/500$ H_2S and KCN on glucose oxidation by vegetative cells of *B. subtilis* I and the spores of all four species investigated. The vegetative cells of *B. subtilis* I are very sensitive to H_2S, while the spores of all four species studied are not sensitive to this reagent, the respiration of the spores of *B. subtilis* I being actually accelerated some 27 % in $M/500$ H_2S. Again vegetative cells were found to be much more sensitive to cyanide than the spores of any of the four species investigated. The spores of *B. megatherium* were the least sensitive to cyanide of all the species studied.

Additional experiments showed that the small endogenous respiration of *B. subtilis* I spores is about 60 % inhibited by $M/500$ KCN and apparently not influenced by H_2S.

Summary.

1. Endospores of aerobic bacilli dehydrogenate certain hexoses and disaccharides anaerobically with methylene blue as hydrogen acceptor. Vegetative cells dehydrogenate glucose more actively than do the endospores which they produce. The velocity of dehydrogenation of glucose by endospores can be greatly accelerated, (*a*) by previous heating of the spore suspensions to 80° for 30 minutes, (*b*) by incubating in the presence of the substrate aerobically for 30 minutes at 40° before adding methylene blue and studying its anaerobic reduction, or (*c*) by a combination of (*a*) followed by (*b*).

2. Both 5 % ethylurethane and a saturated solution of phenylurethane entirely inhibit the activity of the glucose dehydrogenase of vegetative cells, and of heated spores which are not incubated aerobically in the presence of their substrate the same holds true. If, however, heated spores are incubated aerobically for 30 minutes at 40° in the presence of glucose, ethylurethane no longer inhibits the dehydrogenation. Cyanide has no effect on the velocity of dehydrogenation of glucose by spores and somewhat inhibits this reaction in the case of vegetative cells of the same organism.

3. Spores of the four species studied exhibited a small endogenous oxygen uptake, and this was increased some 10 to 40 times by the addition of glucose.

Vegetative cells were found to be considerably more active with respect to their ability to oxidise glucose than their corresponding endospores.

4. A lag period of 50 to 60 minutes was observed before the velocity of aerobic oxidation of glucose by freshly prepared unheated spores became constant. Previous heating at from 60° to 90° for 30 minutes considerably shortened this lag period, but the final constant velocity of oxidation was only very slightly greater in heated than in unheated spores.

5. Endospores resist heating to temperatures as high as 90° for 30 minutes but they still possess active respiratory catalysts. However, the actual aerobic oxidation of glucose by heated spores is a process which is just as sensitive to temperatures above 50° as is the identical reaction in vegetative cells of the same organism. Thus at 55° the velocity of glucose oxidation by spores and vegetative cells is considerably decreased, the difference being that cooling to the optimum temperature of the reaction is followed by an increase in the rate of oxidation in the presence of spores, but not in the presence of vegetative cells.

6. The aerobic oxidation of glucose by both spores and vegetative cells is inhibited by 5 % ethylurethane and saturated phenylurethane.

7. Both H_2S and KCN strongly inhibited the aerobic oxidation of glucose by vegetative cells of one strain of *B. subtilis* studied, while the identical process in the presence of endospores of the same organism was accelerated by H_2S and inhibited by KCN. In the case of the spores of the three other species H_2S had no effect on the above oxidation, while KCN inhibited it. This appears to be the first instance recorded in which H_2S and KCN have not given parallel results with respect to acceleration or retardation of an oxidation proceeding in the presence of living cells.

I wish to thank Miss M. Stephenson and Prof. D. Keilin for advice and criticism and Sir F. G. Hopkins for his constant encouragement during the progress of this work.

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XXI. GROWTH AND REPRODUCTION ON SYNTHETIC DIETS. III.

A NOTE ON FATS.

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In an earlier paper [Hartwell, 1927] experiments were described in which the mother rats frequently died at the end of pregnancy, and their deaths coincided with the failure of the young to be born. This appeared to be closely connected with an alteration of the fat content of the diet, and hence it seemed advisable that further work should be done, using different fats in a synthetic diet.

It is obvious that the older experiments with fat-free diets are of little value, in that the diets used were deficient in the fat-soluble vitamins not then recognised, and any results attributed to the lack of fat were probably due to a deficiency of either vitamin A or vitamin D, or both. Even with modern knowledge and technique it appears to be doubtful whether "fat-free" diets are absolutely fat-free, since Smedley-MacLean [1922] has found that some of the fat in yeast (so frequently used in diets as the source of the B-vitamins) is in some form of combination in the plasma of the cell and is not extracted from it by alcohol and ether. Opinions of nutrition workers as to the importance of fat in the diet appear to vary considerably.

Drummond [1920–21] obtained growth in young rats on a diet in which the only fat was that of the yeast, since the caseinogen and starch had been repeatedly extracted with alcohol and ether. He, therefore, concluded that pure fats were not essential constituents of a diet unless such a minute amount (9 mg. daily intake) played some important rôle in metabolism as do the accessory food factors. Drummond and Coward [1921] also obtained normal growth in rats from weaning to maturity on diets deprived as far as possible of all traces of neutral fats. Osborne and Mendel [1920–21] put forward a similar view. Their rats on a fat-free diet "quadrupled their weight within the usual time" and were as well nourished as the control animals which received liberal amounts of butterfat and lard. These experiments, however, were made on rats of an initial weight of approximately 70 g., so that it is possible that the animals had reserves which they could utilise. Vitamin work has shown the difficulty of exhausting all reserve stores, and it is conceivable that similar reserves of other unknown factors also exist. McAmis, Anderson and Mendel [1929] obtained good, but not optimum, growth on diets of extremely low fat content, and found that the addition of even a small amount of fat produced a beneficial effect. That fats should be regarded as essential constituents of the rat's diet has been demonstrated by Burr and Burr [1929], who found that on a fat-free diet the animals developed a characteristic disease and died. They also found that the addition of 2 % of fatty acids to the diet prevented and also cured the disease. In other papers [1930; 1932] these

workers put forward the view that linoleic and linolenic acids are essential fatty acids and cannot be synthesised by the mammal, in which case it is obvious that fats containing these acids are essential constituents of the diet. Further evidence in favour of this view is afforded by the experiments of Evans and Lepkovsky [1929] working on slightly different lines. These observers were interested in the sparing action of fat on the antineuritic vitamin B, and they conclude that "fats as such exert an important function in the metabolism of the animal other than supplying energy." Later work of these observers [1931] indicates that the improvement is due to fat itself and not to fat-soluble vitamins, since they were able to produce an improvement in growth by the addition of synthetic fats, entirely free from vitamins A, D and E. Experiments with high fat diets also present discrepancies in results. Smith and Carey [1923–24] found that rats on a high fat ration failed to maintain normal growth after 50 days on the diet, but Levine and Smith [1927] obtained normal growth from 30 to 180 g. in rats on a ration containing 86 % of the total calories in the form of fat. It is highly probable that the explanation of these differences lies in the proportion of the other dietary constituents, since balance between the essential factors has been shown to be important.

Hitherto the importance of fat in the diet has largely been ascribed to its vitamin content, but if essential fatty acids are recognised as are essential amino-acids, the rôle of fat *qua* fat, apart from its calorie value, is obviously more important than has been hitherto recognised.

Experimental.

(*a*) *The basal diet* consisted of:

20 g. pure caseinogen (Glaxo).
64 g. pure potato starch.
4 g. salt mixture [Hartwell, 1922].
5 g. marmite.
300 cc. distilled water.

The diet was prepared according to the "cooked" method previously described [Hartwell, 1925]. Food was supplied so that the animals ate as much as they wished; when a very small amount was left the ration was increased and when a large amount remained the ration was correspondingly cut down, so that an approximate food intake could be recorded. Distilled water was given *ad lib.*

(*b*) *Fats.* The following fats were used.

1. Beef suet. The fat was freed as much as possible from connective tissue and minced finely.
2. Mutton suet. (Prepared as for beef.)
3. Bacon fat. Cheap streaky bacon was used, the fat being separated and finely minced.
4. Butter. Fresh dairy butter.
5. Margarine. A well known brand.
6. Lard.
7. Olive oil.

In a preliminary experiment the fat was fed separately from the basal diet, but this proved unsatisfactory, because the amount eaten varied considerably from day to day and the individual growth curves were so widely divergent that no conclusions could be drawn. Since these experiments were started it has been

shown by Kon [1931] that rats do not choose suitably when offered separate food factors.

In a second series of experiments cod-liver oil was omitted from the diet, because previous results had suggested that this factor might be the cause of the maternal mortality [Hartwell, 1927] and also there was reason to suppose that

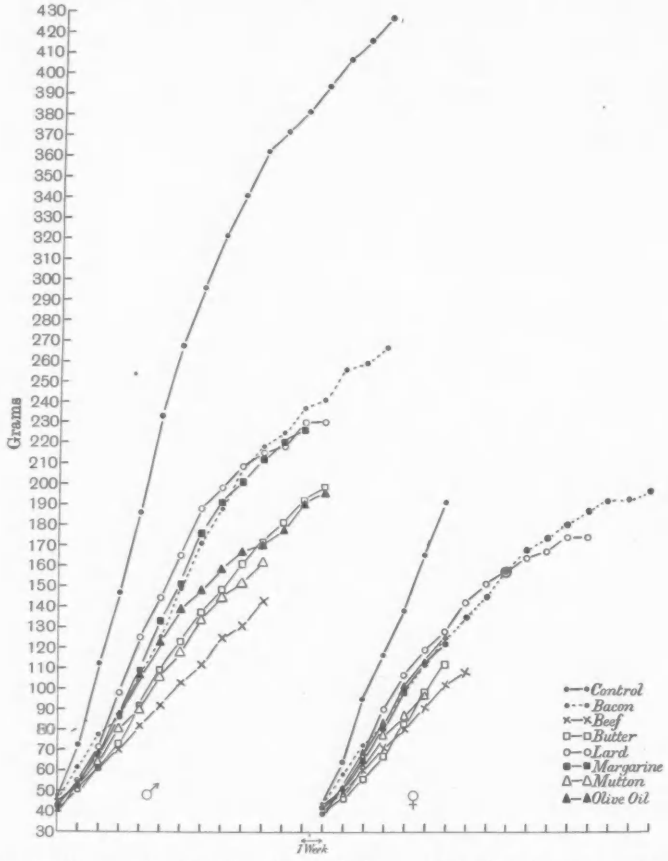

Fig. 1. Growth curves of male and female rats. Each curve is taken from the average of 6 animals.

vitamin A was supplied in the fats used. After 12 weeks on the diet, the rats receiving bacon fat exhibited typical xerophthalmia and 4 of them died. The remaining 8 were given 3 drops of cod-liver oil per day and all of them recovered. Hence the experiments had again to be repeated, and cod-liver oil was added to each diet. In the final experiments (of which the average weight curves are shown in Fig. 1) 16 g. of fat and 4 cc. of cod-liver oil were added to the basal diet, so that the animals were receiving a diet containing approximately 20 % of both protein and fat.

(*c*) *Animals.* Piebald rats bred in the laboratory were used, 12 for each experiment, 6♀ and 6♂, and were kept in large metal cages with screens of such a mesh that all excreta fell through and were not accessible to the animals. They were weighed daily, except Saturdays and Sundays, after the first 4 weeks. The does were removed to separate wooden cages a few days before litters were expected.

Results and comments.

Growth and general condition. In each experiment growth was obtained, but in no case was it equal to that produced by the control animals receiving a mixed diet of kitchen scraps (see Fig. 1). As will be seen from the curves, the rats which were given bacon fat, lard or margarine showed better growth than those eating olive oil, butter or mutton fat, while those fed on beef suet showed the poorest growth of all. With the exception of one doe which died at the birth of her litter, all the rats appeared to be fit and well. They were very active and apart from the condition of their fur could be regarded as healthy animals. In each experiment the fur began to come out after about 10 days on the diet and at the end of the second week many animals had quite large bald patches. The fur eventually grew again, but at different rates, and in some instances had not fully grown at the end of the experiment. The fur of the animals eating lard grew again more rapidly than that of any others. In some animals the skin of the bald patches became brown and scaly; this was especially noticeable in the rats which received olive oil. The loss of fur can hardly be associated with the quality of the fat since it was a feature common to all the experiments, unless each fat were equally deficient. It seems more probable that the deficiency is due to the protein of the diet, possibly to the small amount of cystine in the caseinogen.

Reproduction. No litters were born to does fed on bacon fat or lard. *Post mortem* examinations showed indications of resorptions in the uterus, suggesting a lack of vitamin E in the diet. This is in agreement with the work of Evans and Bishop [1923] who found that lard failed to give normal fertility in a large proportion of female rats. Simmonds, Becker and McCollum [1928] state that certain samples of lard "promote fertility to an extent which compels the conclusion that they contain considerable amounts of E." That bacon fat and lard both failed to produce fertility suggests that the fat of the pig is deficient in vitamin E. A possible explanation of this discrepancy in results is that the rats used by Simmonds, Becker and McCollum had a large reserve of vitamin E and were therefore able to produce litters although the diet contained insufficient of this factor to provide for reproduction. It is interesting that although the rats fed on bacon fat and lard failed to produce young, yet their growth was decidedly better than that of other rats which produced young at the usual time. These experiments, therefore, afford further evidence that dietary factors essential for growth are entirely different from those required for fertility.

The author was unable to find any references comparing the amounts of linoleic and linolenic acids in the fats used, but it is possible that lard and bacon fat contain more of the essential fatty acids than do the other fats and hence give better growth. According to Lewkowitsch and Warburton [1922] lard contains small quantities of linoleic acid and perhaps also linolenic acid, but no definite statement is made as to the presence of these fatty acids in the other fats used in these experiments.

With butter, beef suet, mutton suet, olive oil and margarine litters were born to most of the does, but none of the diets can be regarded as satisfactory for reproduction or lactation. The does became pregnant at different ages and also

at different weights, considerable variation occurring in each experiment. The litters were small and on the whole the young were of low weight compared with those of control animals; they were rarely reared and usually died within a day or two of birth. Of those which survived, the average weight at 21 days was from 19·8 to 28 g. while the young of the control rats weighed from 46 to 49 g. Only one doe died at the birth of her litter; it is therefore unlikely that the fat of the diet was the factor causing the high maternal mortality noted in previous work [Hartwell, 1927]. The experiments were not carried beyond the first litters because the breeding results were so poor that it seemed inadvisable to continue the diets longer.

It is interesting to note that the growth obtained with margarine was as good as that shown with any fat and that it also provided for the needs of reproduction, while with lard and bacon fat no young were born, although the growth curves were equally good.

Summary.

1. With a basal diet of pure caseinogen, potato starch, salt mixture, marmite and a little cod-liver oil, the addition of bacon fat, lard or margarine produced better growth than butter, beef suet, mutton suet or olive oil.

2. Reproduction did not occur in rats fed on bacon fat or lard.

3. Maximum growth was not obtained on any of the synthetic diets used, and reproduction was poor; the young frequently died within a day or two of birth, and those reared were of low weight at weaning.

4. The general condition of the rats fed on these synthetic diets was good except for a characteristic loss of fur.

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XXII. NOTE ON THE PREPARATION AND FRACTIONATION OF THE α-NAPHTHYL*iso*CYANATE COMPOUND OF PLASTEIN.

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(*Received December 19th, 1932.*)

That plastein, a substance precipitated from concentrated solutions of products of peptic hydrolysis of proteins when incubated at p_H 4 with pepsin, is a complex mixture of several substances is indicated by the fact that its compound with phenyl*iso*cyanate can be fractionated [Folley, 1932]. This observation prompted a more extended investigation of fractions derived from plastein by way of its compound with α-naphthyl*iso*cyanate. Unfortunately, circumstances prevented completion of the work. It was considered of interest, however, briefly to record the results that were obtained, since they confirm the conclusion reached previously that plastein is a mixture.

Experimental.

Well washed plastein obtained as a precipitate by incubation at 37° of a 40 % solution of Witte peptone (brought to p_H 4 by addition of HCl and containing 2 % of Merck's pepsin powder) was purified by thrice repeated solution in the minimum quantity of dilute NaOH followed by precipitation with acetic acid. Its α-naphthyl*iso*cyanate derivative was prepared by shaking a 2 % solution in just sufficient NaOH with excess of α-naphthyl*iso*cyanate. The di-α-naphthylurea originating from excess of α-naphthyl*iso*cyanate was filtered off and the plastein derivative precipitated by acetic acid.

From a weakly alkaline 1 % solution of the α-naphthyl*iso*cyanate compound of plastein containing 3 % NaCl, two substances of different basicities were obtained. The first substance was precipitated by saturation of the weakly alkaline solution with CO_2. This fraction was filtered off and the second substance precipitated by addition of acetic acid. By separately subjecting each fraction to repetitions of this process, each was freed from traces of the other, and finally two quite sharply differentiated substances were obtained.

Exhaustive alcoholic extraction of these two substances in the Soxhlet apparatus yielded in each case a substance soluble in warm alcohol and a residue insoluble in alcohol.

Both alcohol-soluble fractions were repeatedly extracted by prolonged shaking with large quantities of cold absolute alcohol. Both yielded as a result of a long series of operations three sub-fractions, namely: a substance soluble in warm absolute alcohol but insoluble in the cold; a substance soluble in cold absolute

alcohol and a small quantity of reddish alcoholic solution containing the most soluble fractions, together with possible decomposition products of the α-naphthyl-*iso*cyanate compound resulting from the treatment with alcohol.

The author wishes to make grateful acknowledgment to Prof. H. S. Raper for his unfailing encouragement and the facilities provided for the commencement of this work, and to Prof. H. J. Channon for making possible its continuation.

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XXIII. THE DETERMINATION OF VITAMIN C IN DIPLOID AND TETRAPLOID TOMATOES.

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In an attempt to find the relationship between the antiscorbutic activity of fruits and the physiological condition of the plants, Bracewell, Zilva and co-workers [1930; 1931] have examined a number of varieties of apples grown under different conditions. Bramley's seedlings were found to be the most potent of the first varieties tested.

Prof. J. B. S. Haldane drew the attention of Crane and Zilva [1931] to the fact that these apples belonged to the triploid group (with 51 chromosomes) whereas the other varieties tested belonged to the diploid group (with 34 chromosomes) and suggested that the high vitamin C content might be associated with the triploid nature of the chromosomes. Two other triploid varieties were, therefore, examined and found to have high antiscorbutic activity though one of them was equalled in potency by one of the diploid group.

Dr F. W. Sansome proposed the tomato as a more suitable fruit for further investigation since the genetic constitution of the apple is complex and apple trees cannot be manipulated easily for genetic purposes. It is comparatively simple to obtain tomatoes with twice the normal number of chromosomes and such tetraploid varieties may be produced directly from diploid plants [Winkler, 1916; Jorgensen, 1928; Sansome, 1930].

The vitamin C content of tomatoes has been examined by many workers. Givens and McClugage [1919] showed that a daily dose of 10 g. would provide complete protection from scurvy, but they did not determine the effect of smaller doses. Delf [1921; 1924] found the minimum protective dose of freshly expressed juice of tomatoes to be 2 cc. for English fruit, and 4 cc. for South African fruit. Höjer [1924] tested juice from fruit which was not fully ripe and found that a daily dose of 3 cc. produced only slight protection (0·1 minimum protective dose in his nomenclature). House, Nelson and Haber [1929] showed that the vitamin C content of the fruit was dependent upon the degree of ripeness and method of ripening. Whereas 4 g. of green tomato produced only slight protection from scurvy, an equal dose of vine-ripened fruit produced complete protection. Since these results show much variation, tomatoes bought in the open market were examined by the method in use in these laboratories to determine a useful dose for test before beginning the investigation of specially grown diploid and tetraploid varieties.

Preliminary experiment to determine the vitamin C content of tomato juice from fruit bought in the open market.

The method described by Key and Elphick [1931] was used to determine the potency of tomato juice. The scorbutic diet consisted of: bran 45 %, crushed oats 25 %, dried milk 30 %, supplemented by cod-liver oil given twice weekly.

The 16 guinea-pigs used for the test were divided into 4 groups each of 4 animals, and were given the basal diet together with the additions shown in Table I.

Table I. *The antiscorbutic potency of tomatoes bought in the open market.*

Group	Dose	Guinea-pig	Degree of protection estimated by K.K.	Degree of protection estimated by F.D.	Average degree of protection for each dose
I	1·5 cc. tomato juice	1	0·5	0·5	1·2
		2	2·0	1·5	
		3	0·5	1·0	
		4	2·0	1·5	
II	3 cc. tomato juice	5	2·0	2·0	1·9
		6	2·0	1·5	
		7	2·0	2·0	
		8	2·0	2·0	
III	1·5 cc. lemon juice	9	3·0	3·0	3·4
		10	4·0	4·0	
		11	3·0	3·0	
		12	3·5	3·5	
IV	No dose	13	1·5	1·0	0·9
		14	1·0	1·0	
		15	0	0	
		16	1·0	1·5	

Twice a week, $\frac{1}{2}$ lb. of small ripe fruit was bought in the open market and stored at 0° until used. Each day one or two tomatoes were cut up, pounded in a mortar and squeezed through muslin. The juice thus obtained was very viscous, and it was necessary to select a pipette with a wide mouth for giving the dose to the animals. In group III, freshly expressed lemon juice was used without decitration. By careful manipulation, the animals could be induced to take the dose with very little, if any, loss. After receiving doses for 14 days, all the animals were killed and their jaw-bones were removed and placed in a decalcifying solution until soft. Transverse sections of each incisor root just in front of the first molar indicated the severity of the scurvy developed in each guinea pig.

Two independent observers classified the animals by reference to the table given by Key and Elphick in which 5 degrees of protection from scurvy are described. In this series, 0 represents no protection and 4 complete protection. The results are shown in Table I from which it will be seen that the average degree of protection produced by 1·5 cc. tomato juice was 1·2 and the corresponding figure for 1·5 cc. lemon juice was 3·4. By reference to the curve relating degree of protection to dose of vitamin C given, the abscissae corresponding to 1·2 and 3·4 degrees of protection were found to be 0·52 and 2·48 respectively.

Therefore, $$\frac{\text{Vitamin C in 1·5 cc. tomato juice}}{\text{Vitamin C in 1·5 cc. lemon juice}} = \frac{0·52}{2·48} = 0·21.$$

Since, by definition, 0·1 cc. lemon juice contains 1 unit of vitamin C, 1 cc. tomato juice contains 2·1 units.

In group II, 3 cc. tomato juice given daily produced an average degree of protection of 1·9 for which the corresponding abscissa is 1·14. Hence 1 cc. tomato juice contains 2·3 units.

This result confirms that obtained from group I and shows that the tomato juice has about one-fifth the potency of lemon juice.

Comparison of diploid and tetraploid tomatoes.

Diploid and tetraploid plants of comparable constitution (*D*, *O*, *P*, *S*, *R* and *Y* in factor symbols) were grown in neighbouring rows at the John Innes Horticultural Institution. Samples of each kind of fruit were gathered twice weekly and delivered to the laboratory on the day of picking. They were stored at approximately 2° until used. The tetraploid tomatoes were all smaller than those of the diploid variety and many of both kinds were not fully ripe. 4 or 5 tetraploid and 2 or 3 diploid fruits were used each day. They were crushed in a mortar and the juice and as much pulp as possible were squeezed through muslin as in the last experiment. The juice was well stirred in order to obtain a representative sample for dosing. Since the preliminary test had shown that tomato juice had approximately one-fifth the potency of lemon juice, a daily dose of 5 cc. of each variety of tomato juice was compared with 1 cc. of lemon juice. The results are shown in Table II. The average degree of protection was found to be 3·25 for

Table II. *The antiscorbutic potency of diploid and tetraploid varieties of tomatoes (1st experiment).*

Group	Dose	Guinea-pig	Degree of protection	Average degree of protection for each dose
I	5 cc. diploid tomato juice	17	4·0	3·25
		18	2·0	
		19	4·0	
		20	3·0	
II	5 cc. tetraploid tomato juice	21	3·0	3·4
		22	3·0	
		23	3·5	
		24	4·0	
III	1 cc. lemon juice	25	0·5	2·9
		26	4·0	
		27	3·5	
		28	3·5	
IV	No dose	29	0	0·75
		30	1·5	
		31	0·5	
		32	1·0	

5 cc. diploid juice and 3·4 for 5 cc. tetraploid juice. Thus there appears to be no difference between the two groups. 1 cc. lemon juice produced an average degree of protection of 2·9, a figure which is slightly lower than that obtained from 5 cc. tomato juice. Reference to the curve shows that the diploid tomato juice contained 2·3 units per cc., whereas the tetraploid tomato juice contained 2·4 units per cc. Both of these results approximate very closely to that obtained in the preliminary experiment on tomatoes bought in the open market.

In this test at least one animal in each group showed complete protection from scurvy and the figures truly representing the antiscorbutic effect in these animals might, therefore, be higher than 4 (if it were possible). Any difference between the two kinds of juice might thus be masked. The experiment was, therefore, repeated using 12 more guinea pigs. Half the animals were given a daily dose of 5 cc. diploid tomato juice and the other half were given a daily dose of 2·5 cc. tetraploid tomato juice. The results are shown in Table III. The ratio of the average degree of protection afforded by the two doses was found to be $\frac{3·5}{2·25}$. By reference to the curve this ratio was found to correspond to a dose ratio of $\frac{2·57}{1·46} = 1·8$, showing that 5 cc. diploid tomato juice contain approximately twice

as much vitamin C as 2·5 cc. tetraploid tomato juice. The two varieties therefore have the same potency.

Table III. *Comparison of diploid and tetraploid varieties of tomatoes (2nd experiment).*

Dose	Guinea-pig	Degree of protection	Average degree of protection for each dose
5 cc. diploid tomato juice	33	3·0	3·5
	34	4·0	
	35	3·5	
	36	3·0	
	37	3·5	
	38	4·0	
2·5 cc. tetraploid tomato juice	39	2·0	2·25
	40	2·5	
	41	1·5	
	42	1·0	
	43	3·5	
	44	3·0	

Discussion.

The fact that there appears to be no difference between the vitamin C contents of diploid and tetraploid tomatoes of the same genetic constitution indicates that the number of chromosomes is not the sole factor controlling the vitamin C content. Dr Sansome suggests that "the genetic constitution of the original diploid may be such that the vitamin content was already saturated and the effect of increased chromosome number was not observable. Original diploid plants of different constitution might, therefore, give different results."

Summary.

Juice from tomatoes bought in the open market was found to contain approximately 2·2 international units of vitamin C per cc.

"Diploid" tomato juice from fruit grown at the John Innes Horticultural Institution contained 2·3 units per cc. and "tetraploid" juice from neighbouring plants contained 2·4 units per cc.

Thus no difference in vitamin C activity could be detected in juice from diploid and tetraploid varieties of similar constitution.

I wish to thank Prof. J. B. S. Haldane for suggesting this investigation and Dr F. W. Sansome for supplying the necessary material. I should also like to offer my grateful thanks to Dr J. H. Burn for his constant interest in the work.

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XXIV. NOTE ON THE TECHNIQUE FOR ISOLATING BASES BY MEANS OF REINECKE'S SALT.

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(*Received December 20th, 1932.*)

Reinecke's salt, $[(NH_3)_2Cr(CNS)_4]NH_4$, is proving to be a valuable precipitant for a variety of basic substances, and its use is likely to extend.

The following experience is therefore deemed worthy of brief record.

During a recent investigation the dried reineckate precipitate (20 g.) from an extract of 25 litres of ox-blood was dissolved in acetone and filtered to remove insoluble impurity. The bases in the acetone-soluble reineckate were examined systematically, and two picrates were isolated in small quantity, 2·22 g. (*a*) and 0·5 g. (*b*) respectively, which could not be recognised as those of any known basic constituent of blood-extracts. Analyses of (*a*) and of the chloroaurate derived from it, and the physical properties of these salts, identified the base as 2 : 2 : 6 : 6-tetramethyl-4-piperidone (triacetoneamine), and (*b*) was similarly found to be β-amino*iso*propylacetone (diacetoneamine) picrate.

Comparison of the salts of the two bases, isolated from the mixture of bases precipitated as reineckate, with authentic specimens confirmed the identifications.

Before concluding that these two substances actually occurred in small amounts in blood the suspicion arose that they might be artefacts, and so indeed they proved to be. This was demonstrated in two ways. Firstly, Reinecke's salt was dissolved in acetone, and by a procedure similar to that applied to the bases from the blood-extract small amounts of diacetoneamine and triacetoneamine were subsequently isolated: and secondly, a search for these bases in another blood-extract by a technique which rendered their artificial production impossible was fruitless.

It should be noted, therefore, that when ammonium reineckate is dissolved in acetone a reaction occurs between solvent and solute with the production of diacetoneamine and triacetoneamine. Consequently the purification of a reineckate precipitate containing ammonium reineckate, such as many extracts of biological origin will yield, by dissolution in acetone will result in the production of the two artefacts which have been detected in the present instance. This complication, and the possibility of others of a similar nature, suggest that the use of acetone as a solvent for reineckate precipitates should be avoided when possible.

Since the identification of the two bases was unquestionable and this note is published solely as a warning to users of Reinecke's salt, experimental details are omitted.

XXV. THE CHEMICAL CHANGES INDUCED IN WOOD BY SATURATED STEAM UNDER PRESSURE.

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(*Received December 20th, 1932.*)

Considerable interest has recently been centred in the fact that under the influence of moderate heat wood is subject to chemical changes which may result in the transformation of part of its carbohydrates into a lignin-like body. The effect is noticeable in oak wood at temperatures as low as 50–55° [Campbell and Booth, 1930] and in silver fir wood at 75–80° [Campbell and Booth, 1931]. O'Dwyer [1931] has shown that with increasingly severe conditions of drying at temperatures up to 105° the hot water and sodium hydroxide extracts of oak wood increase in amount and the yields of hemicellulose A decrease. It is suggested that, under drastic drying conditions, hemicellulose A undergoes decomposition, but the products of the decomposition have not yet been identified. According to Hawley and Wiertelak [1931] a pronounced increase in the apparent lignin content of wood is accompanied by a depletion of its carbohydrates after heating in a sealed tube at 135° for 4 to 8 days. In the case of ash wood the decrease in Cross and Bevan cellulose is almost wholly accounted for by the depletion of its associated pentosans while in the case of Sitka spruce wood the hexosan portion of the cellulose is mainly involved. In a recent paper by Slavik [1932] the results are described of a series of experiments in which samples of dry beech and spruce woods were heated in the presence of air for periods of days at a temperature of 140–145°. These results are only in partial agreement with those of Hawley and Wiertelak [1931] since, although an increase in the lignin content took place in the case of the spruce wood, a slight depreciation of the lignin content was observed in beech wood which had been heated for 9 days. Slavik [1932] also observed a decrease in the methoxyl content of both beech and spruce woods under the experimental conditions stated. Following on the work of Hawley and Wiertelak, Hawley and Harris [1932] have heated samples of hardwood and softwood cellulose in sealed tubes at 135° for periods up to 8 days and obtained a product which they have termed "synthetic lignin." Evidence is cited in favour of the view that, in certain essential particulars, this product is similar to natural lignins. Further evidence is derived from the work of Stam, Semb and Harris [1932] on the absorption spectra of lignin solutions, in which it is shown that the thermal reaction-product of cellulose gives an absorption band practically identical with that of lignin. The so-called synthetic lignin of Hawley and Harris [1932] contains no methoxyl groups and is thus differentiated from the lignin ordinarily isolated from wood by means of concentrated mineral acids.

At this stage it should be emphasised that no attempt has yet been made to explain the mechanism of the thermal reactions described above. For instance

it does not appear to be known whether the presence of a certain amount of water vapour is an essential factor. It has been shown by Aronovsky and Gortner [1930] that when the wood of *Populus tremuloides* is cooked with water its pentosan content is rapidly reduced. In fact these authors describe one experiment in which a sample of wood, cooked with water for 12 hours at 150 lbs. pressure and at a temperature of 186°, was almost entirely denuded of its pentosans. Under similar conditions there was marked decomposition of α-cellulose and although lignin was the most stable wood constituent it appeared to undergo a certain amount of depolymerisation. The decomposition is attributed solely to the action of water and high temperatures.

Experimental.

The object of the present investigation was to determine the effect of saturated steam at various pressures on the chemical composition of both a hardwood and a softwood.

The hardwood material consisted of small pieces of beech wood (*Fagus sylvatica*) measuring approximately $2\frac{1}{2} \times \frac{1}{2} \times 10''$ and the softwood material, consisting of pieces of the same size, was cut from African pencil cedar (*Juniperus procera*) heartwood. Prior to treatment the oven-dry weight of each batch of material was determined from its air-dry weight and the moisture content of controls. Separate batches of from 30 to 40 pieces of wood were open-piled in a flat glass dish and exposed for 4 hours to saturated steam at 20, 40, 60 and 80 lbs. pressure per square inch respectively. On removal from the treating cylinder the material was piled in the laboratory until air-dry. After weighing each batch of steamed wood, samples were removed for moisture content determination and the loss in weight sustained by the complete batch was calculated as a percentage of oven-dry unsteamed wood. Each batch was now converted to 80–100 mesh flour and analysed according to the standard procedure. The analytical data for untreated and treated woods are given in Table I and in each case these are expressed as percentages by weight of oven-dry untreated material.

Table I. *Analysis of wood before and after treatment with saturated steam for periods of 4 hours at various pressures.*

Results expressed as percentages by weight of oven-dry unsteamed wood.

	Beech (*Fagus sylvatica*)					African pencil cedar heartwood (*Juniperus procera*)				
	Original wood air-dry	Steamed wood				Original wood air-dry	Steamed wood			
Steam pressure in pounds per square inch	—	20	40	60	80	—	20	40	60	80
Steam temperature in ° C.	—	109	130	145	156	—	109	130	145	156
Loss due to treatment	—	Nil	Nil	3·98	12·03	—	Nil	1·00	2·21	12·48
Cold water-soluble	0·77	1·83	9·73	13·32	9·11	1·50	7·97	7·79	10·80	6·70
Hot water-soluble	1·79	2·07	11·87	15·76	11·68	3·86	9·52	9·35	10·96	7·68
1 % NaOH-soluble	16·00	17·20	31·80	37·05	36·28	21·22	23·34	21·47	19·60	15·68
Alcohol-benzene (1 : 2) soluble	1·43	1·70	6·11	14·28	19·07	4·56	6·41	8·55	10·64	8·66
Cellulose	62·75	55·34	54·64	47·37	47·00	43·05	39·38	40·89	36·33	35·68
Lignin	22·13	23·52	20·14	18·89	16·53	36·90	41·84	41·03	41·99	37·79
Total methoxyl content	6·11	6·15	6·05	5·77	5·42	5·94	5·83	5·93	5·96	5·95
Methoxyl content of lignin	4·44	4·26	4·17	3·33	2·89	4·82	5·00	4·87	5·08	4·75
Methoxyl content of lignin (as % lignin)	20·05	18·11	20·72	17·61	17·50	13·00	11·95	11·77	12·11	12·43
Total pentosans	25·99	24·85	24·85	21·22	12·62	11·10	10·88	10·83	9·59	6·68
Pentosans in cellulose	18·33	11·96	10·52	5·83	4·62	3·16	2·83	2·60	2·22	1·63
Pentosans not in cellulose	7·66	12·89	14·33	15·39	8·00	7·94	8·05	8·23	7·37	5·05

Examination of the data in Table I indicated that a considerable proportion of the extractives in wood which had been steamed was soluble in both water and alcohol-benzene and the data in Table II were obtained to show the extent of overlapping in beech wood which had been steamed for 4 hours at 60 lbs. pressure.

Table II. *Analytical data showing the extent of overlapping between the water and alcohol-benzene extracts of beech wood steamed at 60 lbs. pressure for 4 hours.*

Results expressed as percentages by weight of original unsteamed wood.

Extracted by water at 100° in 3 hours	15·56
Extracted by alcohol-benzene in 6 hours	13·65
Total	29·21
Extracted by water at 100° in 3 hours followed by alcohol-benzene in 6 hours	19·90
Extracted by alcohol-benzene in 6 hours followed by water at 100° in 3 hours	20·89

Material soluble in both hot water and alcohol-benzene 8·32 to 9·31

It has been indicated in previous work that the lignin-like body formed during the heating of hardwoods is formed from pentosans, while in the case of softwoods the parent substance is a hexosan. No results have hitherto been obtained however to show whether any of the wood polysaccharides are converted into simple sugars in the course of pyrolysis. The following experiments were carried out with the object of gaining some information on this point. In the first place 2 g. samples of steamed and unsteamed wood were extracted in duplicate with 100 cc. of water at 100° for 3 hours. After filtration, each residue was washed with 100 cc. of hot water, and the washings were added to the extract. The pentosan content of each oven-dry residue was then determined by the phloroglucinol method. Reducing sugars were estimated colorimetrically in one of each pair of diluted aqueous extracts by means of the Benedict-Lewis picric acid method in the modified form recommended by Willaman and Davison [1924] (Table III). In order to obtain reliable values for total sugars, it was found necessary to adopt the further modification of the above method employed by Aronovsky and Gortner [1930] which involves a preliminary hydrolysis with 2 % HCl at 100° for 2 hours.

Table III. *Analytical data showing the effect of steaming under pressure on the pentosans of wood and the sugar content of the hot water extracts.*

Results expressed as percentages by weight of oven-dry unsteamed wood.

	Beech (*Fagus sylvatica*)					African cedar (*Juniperus procera*)				
	Original wood	Steamed for 4 hrs. at 20 lbs. pressure	Steamed for 4 hrs. at 40 lbs. pressure	Steamed for 4 hrs. at 60 lbs. pressure	Steamed for 4 hrs. at 80 lbs. pressure	Original wood	Steamed for 4 hrs. at 20 lbs. pressure	Steamed for 4 hrs. at 40 lbs. pressure	Steamed for 4 hrs. at 60 lbs. pressure	Steamed for 4 hrs. at 80 lbs. pressure
Total furfuraldehyde-yielding substances calculated as pentosans	25·99	24·85	24·85	21·22	12·62	11·10	10·88	10·83	9·59	6·68
Hot water-soluble	1·83	2·40	12·08	15·99	12·08	4·34	9·26	8·97	10·84	8·30
Pentosan content of wood residue after extraction with hot water	25·39	23·63	16·70	12·19	6·93	10·83	8·40	6·64	5·94	4·37
Furfuraldehyde-yielding substances in hot water extract, calculated as pentosans	Nil	Trace	7·57	9·55	4·63	Nil	2·14	3·14	3·18	2·39
Total sugars in hot water extract	1·59	2·38	10·16	13·00	8·79	3·78	9·13	9·16	11·06	7·14
Reducing sugars in hot water extract	Nil	Trace	4·90	8·95	7·58	3·13	5·96	6·65	8·94	6·31

The furfuraldehyde-yielding bodies were then determined in the remaining aqueous extracts as follows. To 66·3 cc. samples of the respective extracts 33·7 cc. of hydrochloric acid of sp. gr. 1·16 were added drop by drop in order to obtain 100 cc. of a solution of the extractive in approximately 12 % HCl. This was distilled in the ordinary way and furfuraldehyde was determined as the phloroglucide. For comparative purposes the results were expressed as pentosans using Kröber's tables (Table III).

Discussion.

A review of the results in Table I reveals that they have many points in common with those of Hawley and Wiertelak [1931] on the one hand and those of Slavik [1932] on the other. The common factor of the three separate investigations is temperature, for, in the case of softwoods at least, the starting material may be heated in sealed tubes, in the presence of air, or in the presence of saturated steam with essentially the same order of result as revealed by current methods of analysis. The results of the present investigation appear to depict a new aspect of the thermal decomposition of wood, however, since, in the absence of any direct evidence to the contrary, it must be concluded that the presence of excess water vapour at the outset catalyses the primary reactions to the extent that stages hitherto only reached in a period of days at temperatures of 135° and 140–145° can be attained in a few hours at much lower temperatures. A further important point to be deduced from Table I is that although under certain conditions steaming may only induce a negligible loss of wood substance, it none the less gives rise to the same order of chemical change which has been observed by the other workers mentioned above. It is only at the higher steam pressures and temperatures that loss in weight becomes considerable. The analytical data therefore appear to supply a much closer insight into the mechanism of the thermal decomposition of wood than has hitherto been available. In so far as the major constituents are concerned, the mechanism appears to be essentially the same in both the hardwood and the softwood. In both cases the primary decomposition takes place in the Cross and Bevan cellulose although the main objectives are pentosan units in the hardwood and hexosan units in the softwood.

The results given in Table I are sharply differentiated from those of previous workers by the fact that, until the higher temperatures are reached, the total pentosan contents of the hardwood and softwood respectively suffer only slight depletion despite the observed fact that there is depletion of the pentosans in the cellulose, which is naturally most marked in the hardwood. It would further appear that steaming has brought about the transference of a portion of the pentosans from one category to another.

It is observed from Table I that in the case of beech wood an increase in lignin content is only observed after the 20-lb. treatment and that this increase is almost wholly accounted for by the loss in total pentosans. This loss can in turn be traced to the pentosans in the cellulose. It is therefore obvious that, under the conditions stated, only a small portion of the pentosans split off from the Cross and Bevan cellulose gives rise to a lignin-like substance and it is of interest to examine the data in Table III in order to determine in more detail the effect of the steaming treatment on the pentosans as a whole.

In the first place it can be seen that the hot water-soluble material of the unsteamed beech wood consisted largely, if not entirely, of a polysaccharide, and since no pentosans could be detected it must presumably have been a hexosan.

The aqueous extract of beech wood steamed at 20 lbs. pressure was also composed mainly of hexosans although a trace of furfuraldehyde was detected after

distillation with 12 % HCl. It is therefore indicated that the greater part of the pentosans split off from the Cross and Bevan cellulose during steaming at the above pressure occurs as such in the wood residue. It has been seen previously that a comparatively small portion is probably converted into a lignin-like substance which is insoluble in 72 % sulphuric acid. After the 40-lb. treatment an amount of "pentosan" corresponding almost exactly to the amount split off from the Cross and Bevan cellulose (Table I) can be extracted from the wood with hot water. It is observed that the aqueous extract contains reducing sugars but even after allowing for the presence of approximately 2·5 % of hexoses it must be concluded that, during steaming, part of the pentosans is hydrolysed to pentoses. At this stage there is decomposition of lignin (Table I) and since there is no loss of weight it must be concluded that soluble products are formed from this constituent. In this connection be it observed that the sum of the total sugar content of the hot water extract (Table III) and the loss of lignin is approximately equal to the value obtained for hot water-soluble material.

After the 60-lb. treatment beech wood (Table I) loses 3·98 % of its oven-dry weight and it can be seen that this loss can be accounted for by the formation of volatile products from pentosans. At this stage there is decomposition of both parts of the Cross and Bevan cellulose as well as a loss of total pentosans and of lignin. It would appear from the results in Table III that a considerable part of the pentosans must be hydrolysed to pentoses during the 60-lb. treatment but the extent of hydrolysis cannot readily be determined, owing to the presence of hexoses. The comparatively high total sugar content of the aqueous extract suggests that, in addition to the hexosans other than cellulose already accounted for in the original wood, sugar residues have been recruited from the hexosan portion of the Cross and Bevan cellulose. As in the case of the 40-lb. treatment the value for hot water-soluble material closely approximates to the sum of the total sugars (Table III) and the loss of lignin (Table I). The data in Table II indicate that much of the material which is soluble in water is also soluble in alcohol-benzene. The loss in weight caused by the 80-lb. treatment (Table I) can be accounted for by decomposition of pentosans and of these chiefly the pentosans in the cellulose. Further, it is apparent from Table III that the greater part of the furfuraldehyde-yielding bodies remaining in the wood residue are in the form of pentoses. The total sugars obviously include hexoses formed from the Cross and Bevan cellulose.

From the foregoing considerations it would appear that when a hardwood is subjected to the action of steam, under increasingly severe conditions, the first stage in the decomposition consists in the splitting off, as such, of part of the pentosans in the cellulose. It is reasonable to assume that increments to the lignin can only be obtained when the experimental conditions are such that these pentosan units can condense to a product which is insoluble in concentrated mineral acids. If, however, as can be seen from the accompanying tables, the pentosan units in question undergo hydrolysis there is no increase in the apparent lignin content. It is reasonable to assume that the temperature at which the tendency towards hydrolysis of depolymerised units is greatest will vary according to the conditions under which a particular sample of wood is heated since it is only on this basis that the present series of results can be reconciled with those of previous workers. From the results in Table I it can be seen that, when beech wood is heated in the presence of steam, hydrolysis of the pentosan units split off from the cellulose has its inception at a temperature between 109 and 130°. At this latter temperature decomposition of the original lignin itself takes place so that it cannot readily be determined whether the change of pentosan units to

an insoluble product actually ceases at this stage, but the possible extent of such a change must manifestly be small. Since Hawley and Wiertelak [1931] observed additions to the lignin of a hardwood after heating in a sealed tube at 135° it is obvious that under these experimental conditions the temperature limit for the change from pentosan units to a lignin-like body had not been exceeded. On the other hand the results obtained by Slavik [1932] would appear to show that when a hardwood is heated in the presence of air at 145° additions to the lignin are definitely precluded as a result of the hydrolysis and partial decomposition of pentosan units which might otherwise have undergone changes of a different order at a considerably lower temperature. From the results in Table I it may be deduced with reasonable certainty that in the thermal decomposition of wood by steam the first major components to yield volatile products are the pentosans and that hydrolysis of these bodies is an intermediate stage in the process.

Turning now to the consideration of the softwood it can be seen from Table I that consistent marked increases are observed in the apparent lignin content after each of the 20-, 40- and 60-lb. treatments. It is also observed that the total methoxyl content of the wood remains constant throughout the entire pressure-temperature range. There is also little change in the proportion of methoxyl present in the lignin, although during the 80-lb. treatment there is only a slight increase in this latter constituent. These results point to the conclusion that, as in the case of the hardwood, there is a maximum temperature above which additions to the lignin do not take place. It is apparent that the material which under the less severe conditions becomes associated with the lignin is much less stable than the lignin itself at 156°, a fact that might well be taken to indicate that it should not be termed true lignin. It has already been seen that the main source of the increments to the lignin of the softwood is the hexosan portion of the Cross and Bevan cellulose, but reference to Table I shows that at steam pressures up to 40 lbs. per square inch the loss of cellulose is manifestly insufficient to account for the full extent of the increments to the lignin, so that unless part of these increments is recruited from material which is not reckoned in the ordinary wood analysis it must be assumed that in some samples of steamed wood a certain amount of material is estimated both as lignin and as cellulose. Hawley and Wiertelak [1931] have drawn attention to a similar set of conditions for wood heated in sealed tubes. This of course implies that the whole of the material isolated along with lignin from such samples cannot be soluble in 2 % sodium sulphite solution after chlorination and that it cannot therefore be identical with lignin in the accepted sense. It can be seen that only in the case of the 60-lb. treatment is there approximate agreement between the loss sustained by the hexosan portion of the cellulose on the one hand and the observed increase in lignin content on the other.

The analytical data reveal that the pentosans of the softwood behave in a manner similar to those of the hardwood. Steaming up to 40 lbs. pressure brings about a slight increase of "pentosans" not in the cellulose at the expense of pentosans in the cellulose, while at the higher steam pressures there is definite decomposition of part of these bodies. At the same time it is observed from Table III that a portion of the furfuraldehyde-yielding complexes in steamed wood can be extracted with hot water. Joint consideration of the data in Tables I and III affords a clear indication of the source of the increments to the water-soluble fraction of the softwood after steaming. In the first place it must be assumed that the hot water extract of the original wood consists largely of hexoses. After steaming there is a pronounced increase in the amount of total sugars in the aqueous extract of the wood. Part of this increase is obviously

accounted for by products formed from pentosans, but the greater part must be due to the presence of hexosans. Reference to Table I shows clearly that at steam pressures up to 40 lb. per square inch these hexosans cannot be derived from any of the major constituents estimated in the analysis of the original wood, so that it must be assumed that steaming has brought into a soluble form some wood polysaccharide other than cellulose which is not estimated in the ordinary analysis. This view is substantiated by a comparison of the totals, for unsteamed and steamed wood respectively, of hot water-soluble material, cellulose, lignin and pentosans not in the cellulose, after making due allowance for the material in steamed wood which is estimated both as "pentosan" and water-soluble material.

After the 80-lb. treatment the carbohydrates in the aqueous extract of the softwood are present largely as reducing sugars but the total sugar content here is much less than that observed after the less severe treatments. When it is borne in mind that at this stage the greater proportion of the material split off from the Cross and Bevan cellulose does not become associated with the lignin it can only be assumed that the large observed loss of weight (Table I) is accounted for by partial decomposition into volatile products of Cross and Bevan cellulose, pentosans not in the cellulose and polysaccharides other than the foregoing which are not normally estimated in wood analyses.

Summary.

When wood is heated by means of saturated steam under pressures ranging from 20 to 80 lbs. per square inch, it appears to undergo the same order of chemical change as when heat is applied by other means, but the initial process takes place more rapidly and at temperatures as low as 109°.

The greater part of the residues consists of pentosans in hardwoods and hexosans in softwoods. Depending upon the conditions under which the wood is heated, there is a temperature below which the residues can condense to a product which is insoluble in 72 % sulphuric acid and above which they are more or less completely hydrolysed to reducing sugars. Part of the reducing sugars may even be decomposed into volatile products. The temperature at which the tendency towards hydrolysis of depolymerised units is greater than the tendency towards condensation to a lignin-like product is lower for hardwoods than for softwoods. On the basis of the foregoing considerations, the results recorded above can be reconciled both with those of Hawley and Wiertelak [1931] and of Slavik [1932].

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XXVI. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

V. THE EFFECT OF ADRENALINE ON THE DISTRIBUTION OF GLYCOGEN IN THE ALBINO RAT.

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As indicated in previous papers [Hynd and Rotter, 1932, 1, 2], á series of investigations has been in progress in this laboratory for some time on the distribution of fat and glycogen, which follows the administration of certain of the endocrine secretions. The present paper deals with the results obtained in a study of the effect of adrenaline on the liver- and muscle-glycogen of the albino rat under two distinct nutritional conditions, namely (i) a carbohydrate-rich diet of bread and milk, and (ii) a carbohydrate-free diet consisting of cheese.

Experimental.

The methods of analysis of the blood-sugar, liver- and muscle-glycogen, and liver-fat were the same as previously described [Hynd and Rotter, 1930]. Young animals, the majority females, of approximately 100 g. body weight at the time of the experiment were used. The dose employed was 3 minims of 1/1000 adrenaline chloride (Parke, Davis and Co.) per 100 g. rat. The hormone was administered subcutaneously at definite intervals before killing the rat by stunning, so that time-curves of the results could be plotted. This method of killing an animal is held by certain authors [Sacks, 1931] to lead to low values for muscle-glycogen. The values obtained in the present series of experiments, however, do not bear this out. Moreover, as comparisons are always made with control animals under similar nutritional conditions but receiving no injection, the results, though they may not represent the actual *ante mortem* glycogen content of the muscles, give a good index of the changes brought about by administration of adrenaline.

Results.

Under the above dosage of adrenaline the animals are soon markedly affected. They become excited and irritable, appear to find it impossible to get into a comfortable position, and consequently they are very restless. Frequently they lose control of their hind-limbs. As a rule the excretion of urine is frequent and copious. The symptoms appear maximum at $\frac{1}{2}$–1 hour after the injection.

The average results of the chemical analyses are summarised in Table I A and B.

Table I. *Analyses of rats after the subcutaneous injection of adrenaline chloride (3 minims 1/1000 per 100 g.).*

Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
		A. Carbohydrate-fed.			
0	0·15	0·55	3·5	3·7	120–140
¾	0·265	0·47	2·32	3·3	149
1	0·286	0·79	5·21	4·23	106·5
1	0·536	1·3	3·24	5·25	112
1	0·339	0·78	5·6	—	—
1½	0·34	0·43	2·55	3·17	106
2½	0·325	0·52	1·85	3·6	116
3	0·338	0·34	0·92	4·95	127
5	0·43	0·345	1·11	3·5	123
6	0·21	0·37	1·52	3·9	122·5
8	0·148	0·42	1·8	3·9	146
12	0·146	1·23	4·5	4·7	117
14	0·17	—	4·7	3·7	128
15	0·152	1·50	7·25	4·8	109
15¾	0·113	1·25	9·25	3·98	—
17	0·153	0·74	6·66	3·6	138
17½	0·136	1·2	5·55	4·0	124
18	0·16	1·1	5·25	7·2	113
19	0·17	1·71	4·39	3·16	117
21	0·15	0·85	3·4	2·96	156
		B. Non-carbohydrate-fed.			
0	0·17	0·63	1·9	5·0–7·0	100–128
¾	0·38	0·71	1·9	9·7	80
1	—	—	—	4·0	113
1½	0·36	0·30	0·73	5·66	118
2	0·367	1·20	1·26	6·77	101
2¼	0·39	0·55	0·89	7·23	96·5
6	0·33	0·33	1·39	6·30	117
8	0·145	0·33	2·1	7·50	118
15	0·156	0·62	2·75	4·52	87·8
20	0·165	0·80	2·60	6·5	101
24	0·155	1·36	1·2	3·1	—

Blood-sugar. In both carbohydrate-fed and non-carbohydrate-fed rats a distinct hyperglycaemic state is soon evident and continues for about 6 hours. A normal level is reached about the 8th hour, and there is a tendency to slight hypoglycaemia at the 15th hour.

Liver-glycogen. In three instances[1] a rise in liver-glycogen was detected at the 1st hour, corresponding to that described by Cori and Cori [1928, 1]. However, in all the other experiments on carbohydrate-fed rats, a decided decrease in liver-glycogen content occurred during the initial 3–4 hours. After diminishing to the normal fasting level about the 4th hour, the liver-glycogen then increases steadily to a maximum of over 9 % at the 16th hour. Thereafter a very rapid fall to the normal level occurs.

In non-carbohydrate-fed rats the liver-glycogen falls rapidly, reaching a minimum value of 0·73 % after 1½ hours; increases somewhat slowly to a maximum of 2·75 % at the 15th hour, and then returns to the level for control cheese-fed animals.

Muscle-glycogen. The muscle-glycogen of carbohydrate-fed rats, though somewhat irregular during the initial period, falls to a minimum of 0·34 % about the 3rd hour, and remains close to this value until the 8th hour, when it increases,

[1] These values are given in Table I, but are not plotted in Fig. 1.

reaching a maximum of 1·5 % at the 15th hour, that is three times the initial value. A slow fall to the normal value ensues. In the case of carbohydrate-fed animals, changes in muscle-glycogen are more or less synchronous with changes in liver-glycogen. With non-carbohydrate-fed rats, on the other hand, changes in muscle-glycogen tend to lag behind the liver-glycogen changes. Thus, whereas the liver-glycogen passes from a minimum at the 2nd hour to a maximum at the 15th, the muscle-glycogen remains at a low level for a long period, from the 2nd to the 8th or 9th hour, and thereafter increases very slowly.

Liver-fat. The values found for liver-fat were somewhat irregular, but the average value was not far removed from that obtained for animals under similar dietetic conditions but which had not been injected with adrenaline.

Average percentage of fatty acids in liver.

Rats	Carbohydrate-fed	Non-carbohydrate-fed
Not injected	3·7	6·0
Injected with adrenaline	4·105	5·98

Hence this hormone exerts no definite effect on the liver-fat, a finding corroborated by Geiger and Schmidt [1928] and consistent with the recent work of Long and Venning [1932], who have shown that adrenaline has probably no effect on blood-fat.

Discussion.

If consideration be given to the time relationships, the foregoing results are on the whole in agreement with the current views on the effect of adrenaline on carbohydrate metabolism [Cori, 1931]. Nevertheless, in certain respects the results now reported are at variance with the findings of other workers. Reasons for these discrepancies will be suggested in the subsequent discussion.

It is universally admitted that adrenaline causes hyperglycaemia, and it has been demonstrated by experiments on hepatectomised animals [Mann and Magath, 1922; Soskin, 1927] that for its occurrence the presence of the liver is essential. But the degree of hyperglycaemia does not depend on the amount of glycogen already stored in the liver. Thus, in the series of experiments with cheese-fed rats, having an average liver-glycogen of 1·9 % at the time of injection, a greater rise in blood-sugar ensues after adrenaline than in the case of carbohydrate-fed rats with an average liver-glycogen of 3·5 %: and this holds not only at the beginning of the experiment but also during the entire period of 6 hours during which hyperglycaemia is evident.

	Carbohydrate-fed rats		Non-carbohydrate-fed rats	
	Initial value	Average value during hyperglycaemic phase	Initial value	Average value during hyperglycaemic phase
	%	%	%	%
Blood-sugar	0·15	0·331	0·17	0·365
Liver-glycogen	3·5	2·584	1·9	1·235

This behaviour is also consistent with the observations of Abderhalden and Wertheimer [1924], who have shown that, as regards its power to produce hyperglycaemia, adrenaline is more effective in rats receiving no preformed carbohydrate in their diet than in control animals. Moreover, Cori and Cori [1928, 1] have shown that mobilisation of the glycogen present in the liver at any one time

would fail to account for all the extra glucose appearing in the circulation. It is well established, therefore, that this effect of adrenaline cannot be attributed entirely to a conversion of preformed liver-glycogen into blood-glucose.

Nevertheless, during the hyperglycaemic phase the most characteristic effect on the liver-glycogen is a decrease. In both nutritional conditions studied, the liver-glycogen only begins to increase when the blood-sugar shows a return to the normal level. Working with fasting young rabbits, Sahyun and Luck [1929] found the liver-glycogen to fall during the 1st hour and a half, while Cori and Cori [1928, 1, 2, 3] in their experiments with rats in the post-absorptive state observed not a fall but a rise in liver-glycogen. Later, however, Cori, Cori and Buchwald [1930], while admitting that in the first 15 minutes of adrenaline action the liver-glycogen fell from an average of 2·44 to 1·69 %, claimed that the demonstration of a fall in liver-glycogen in the rat is possible only shortly after the injection because at that time glycogenolysis is rapid while the opposing process of glycogen synthesis has not attained its full rate. They ascribe the different behaviours of the two species to the fact that the metabolic changes are much more rapid in rats than in rabbits. However, in view of the results now reported and also those of Eadie, which will be referred to below, it seems that other factors, in addition to the difference in species, must be taken into account.

Eadie [1929; 1930] obtained a definite increase in liver-glycogen and an equally definite decrease in that of the muscle in experiments with rats 1 hour after the injection of a small dose of adrenaline (0·1 mg. per kg.). He thus confirmed the results reported by Cori and Cori [1928, 2; 1929], who used 0·02 mg. per 100 g. rat, a dose which they claim is non-glycosuric and hence within the physiological limits [Cori, Cori and Buchwald, 1930]. Further confirmation of this effect on the glycogen distribution has been furnished by Evans and his co-workers [1931]. They observed 3½–4 hours after the subcutaneous injection of adrenaline (0·2 mg. per kg.) into rats, an increase in blood-sugar and in liver-glycogen, and a fall in muscle-glycogen.

On the other hand, in another series of experiments on rats injected with a large dose (1 mg. per kg.), Eadie found that (1) the liver-glycogen 1½ hours after injection never showed an increase, but always a decrease, and (2) the muscle-glycogen did not usually show a decrease in 20 minutes, but might do so later. The latter findings are in full agreement with our results for rats injected with a very large dose of adrenaline (1·8 mg. per kg.). Equally convincing are the results of experiments on young, fasting rabbits, reported by Corkill [1930]. With a dose of adrenaline too small to cause glycosuria (0·15 mg. per kg.) the liver-glycogen increased in 2 hours to 4–5 times the control value, and the muscle-glycogen diminished to about one half that in the controls. On the other hand, adrenaline given in such a dose that it must have been acting in substantial excess (0·5 mg. per kg. by Sahyun and Luck [1929], and 0·75 mg. per kg. by Corkill) caused, 1½–2 hours after injection, not storage but discharge of glycogen from both liver and muscles. It should be mentioned perhaps that in the cat Eadie found both large and small doses of adrenaline to be followed by a decrease of glycogen in the liver and usually by no change in the muscles. He attributed this to a definite species difference, though amytal may have been a complicating factor as Evans *et al.* [1931, 1] have shown that this anaesthetic reduces the liver-glycogen and hence is unsatisfactory for such investigations [1931, 3]. It may be recalled, however, that the present authors found kittens to show a species difference with regard to their response to insulin [Hynd and Rotter, 1931]. Accordingly, though Cori [1931] has questioned the abnormal behaviour of the cat towards adrenaline, it is nevertheless clearly demonstrated that in both the rat and the rabbit the

effects produced by adrenaline are determined to an important degree by the size of the dose employed. This view is supported, to some extent, by the work of Evans *et al.* in the case of the decapitated cat. They have shown that the recovery of liver-glycogen which normally occurs in the course of a few hours after decapitation was not impaired when small doses of adrenaline (0·21 mg.) were given subcutaneously [1931, 2], but was converted into a fall with large doses (1·0 mg. per kg.) [1931, 3].

As the accumulation of liver-glycogen which takes place during the first few hours after a small dose of adrenaline is accompanied by a diminution of muscle-glycogen, it has been suggested that a transference of glycogen from the muscles to the liver takes place, and according to Cori and Cori [1931] lactic acid and hexosemonophosphate are the intermediate substances involved in the change.

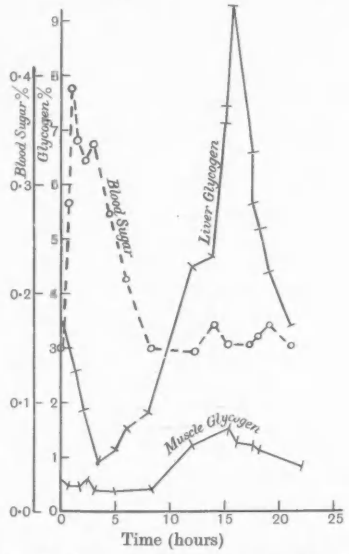

Fig. 1. Effect of adrenaline on the blood-sugar, and liver- and muscle-glycogen of carbohydrate-fed rats.

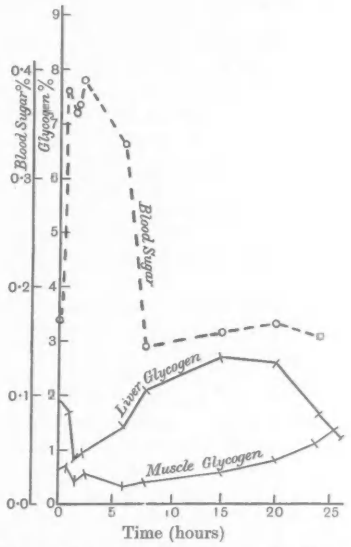

Fig. 2. Effect of adrenaline on the blood-sugar, and liver- and muscle-glycogen of non-carbohydrate-fed rats.

But if this were the correct explanation, one would expect with a large dose a greater augmentation of liver-glycogen. Experiment shows that there is instead a depletion of both liver- and muscle-glycogen during the first few hours. Hence the significant change is not one of glycogen redistribution, as advanced by Cori [1931] but of glycogen breakdown.

But it is important to note that the above mentioned mobilisation of liver- and muscle-glycogen is not the only effect which follows the administration of a large dose of adrenaline. It is to be regarded as the first phase. When observations are continued over a period of 24–30 hours, as carried out by Sahyun and Luck [1929] with rabbits and by ourselves with rats, a second phase occurs, during which glycogen changes in the opposite direction are evident.

From the graph in Fig. 1 it is clear that the liver-glycogen passes from a minimum to a maximum between the 3rd and 16th hours. The muscle-glycogen

remains low until the 8th hour, after which a very definite increase occurs, a maximum being also reached at the 15th hour after injection.

Sahyun and Luck found the liver-glycogen to attain a maximum at the 17th hour, but this close agreement is probably purely fortuitous, and it is to be noted that these workers failed to observe any indication of an increase in muscle-glycogen. This difference is probably due, at least in part, to the fact that whereas they experimented with fasting young rabbits, our work was carried out on rats which had not been deprived of their usual food. Consideration of our results with cheese-fed rats, represented graphically in Fig. 2, supports this view.

The initial fall in glycogen is more rapid, but the levels reached are practically identical in the two series of experiments. The increase in liver-glycogen during the second phase is less marked but quite definite and again attains a maximum at the 15th hour. The muscle-glycogen, though increasing from the 8th hour onwards, does not reach the initial value until the 15th–16th hour. Lack of preformed carbohydrate in the diet seems to be responsible, directly or indirectly, for these differences. Eadie [1930] has made a similar suggestion. Perhaps it should be emphasised that when we state that an increase in muscle-glycogen is observed after the administration of adrenaline, such an increase occurs between the 12th and 21st hours after injection. Further, at the 3rd hour, which is the time usually taken by other observers, little change, or perhaps a slight decrease, in muscle-glycogen was evident in our experiments. Accordingly our results, as already mentioned, are consistent with those of other workers, but further work will be necessary before the details of the changes produced by adrenaline upon the glycogen reserves are finally and completely elucidated.

Certain authors have suggested that the injection of pitressin leads to a stimulation of the suprarenals, so that the effects produced after its injection are in reality due to a hypersecretion of adrenaline. Experiment, however, does not support this view as far as the glycogen changes are concerned. We have shown [Hynd and Rotter, 1932, 2] that the effect of pitressin on the liver-glycogen can be diminished greatly by the simultaneous administration of pitocin. On the other hand, pitocin though given in large amount (16 minims of pitocin per 100 g. rat) along with adrenaline (3 minims) did not inhibit the changes in liver-glycogen. This finding therefore is consistent with the recent observations of Bischoff and Long [1930] that pituitrin or pitressin does not function through stimulation of adrenaline liberation.

Nevertheless, apart from the fact already indicated that adrenaline had no definite influence on the liver-fat, the complete sequence of changes observed to ensue after the administration of this hormone bears a striking resemblance to those already reported by us [Hynd and Rotter, 1932, 1] to result from the injection of pitressin. Comparison of the liver-glycogen curves shows that they almost coincide, the main difference being that where a fall in liver-glycogen is observed, it is always more rapid with adrenaline than with pitressin. Further, though the initial change in muscle-glycogen after pitressin is in the opposite direction from that observed after adrenaline, the changes during the second phase are very similar in character.

The question again arises as to whether the effects observed during the second phase, which is not completed until 25 hours after the injection, are to be attributed to the injected adrenaline, or considered as secondary effects. We are inclined to favour the latter view and to suggest that the true adrenaline effect occurs during the first phase, when glycogen breakdown is in excess of glycogen synthesis, so that there is evident a rise in blood-sugar accompanied by a rapid fall in both liver- and muscle-glycogen [Molitor and Pollak, 1930]. This is consistent with the observations of several authors, including Cori [1930], Bischoff

and Long [1930], and Evans *et al.* [1931, 3], who concluded that subcutaneously administered adrenaline requires 4–6 hours for absorption. Further, Cori found that the blood-sugar continues to rise or remains elevated only so long as there is adrenaline in the blood. Our experiments indicate that the increase in liver-glycogen is accompanied by a fall in blood-sugar to the normal level. Accordingly the synthesis of hepatic glycogen, though the most striking change observed, is probably not due to adrenaline itself but rather to some other hormone or hormones, the secretion of which is stimulated by the primary effect of adrenaline.

Further, an animal with a lowered alkali reserve shows an increased sensitivity to adrenaline [Abderhalden and Wertheimer, 1924]. Consequently rats fed on a carbohydrate-free diet would be more responsive to adrenaline than carbohydrate-fed animals. Experiment shows that while the initial rise in blood-sugar and fall in liver- and muscle-glycogen were more rapid in the non-carbohydrate-fed rats than in the controls, the subsequent rise in liver-glycogen was delayed and less marked with non-carbohydrate-fed animals. This furnishes further evidence that while the changes during the first phase probably are true adrenaline effects, those occurring during the second phase are not. As Horsters [1930] has claimed that in adrenaline hyperglycaemia the quantity of insulin which can be extracted from organs is increased, the second phase effects may be largely dependent on pancreatic activity. The very definite increases in muscle-glycogen then observed suggest that the pancreas has been stimulated and that the resulting insulin has produced one of its characteristic effects, namely re-synthesis of muscle-glycogen [Best *et al.*, 1926; Debois, 1931]. The simultaneous accumulation of glycogen in the liver is somewhat more difficult to account for, as in normal animals, except young rabbits [Goldblatt, 1929; Corkill, 1930], insulin tends to bring about a diminution of liver-glycogen. However, it must not be overlooked that after an excessive dose of adrenaline an animal is for a time in an abnormal condition: its endocrine balance has been upset: there has been hyperglycaemia and glycosuria, and both liver- and muscle-glycogen have been reduced to low levels. In other words, the animal at this stage resembles the diabetic. As it is well known that insulin increases the liver-glycogen in the case of a diabetic animal, it is not altogether improbable that the increase in liver-glycogen observed in our experiments may also be attributed to the pancreatic hormone.

At the same time, insulin may not be the only hormone which complicates the effects produced by adrenaline. Thus, Britton and Silvette [1932] recently reported that while adrenaline failed to augment liver-glycogen, extracts of suprarenal cortex had a marked influence on carbohydrate metabolism, in that the blood-sugar, liver- and muscle-glycogen were all rapidly (within 1 hour) increased, and remained for a considerable period (6 hours) higher than controls. It is possible, therefore, that in our experiments the hormone of the suprarenal cortex may have been exerting an important influence. At any rate it seems established that the effects of an excessive dose of adrenaline on the normal intact animal are not limited to the action of adrenaline itself, but must involve, to some extent, the action of other ductless glands. As we are engaged with experiments to determine which of these glands are implicated, further discussion of this point would be premature.

SUMMARY.

The effects produced by adrenaline vary with the size of the dose employed.

In large dosage, adrenaline produces in rats, on either a carbohydrate-rich or a carbohydrate-free diet, striking changes in the blood-sugar and in the

glycogen content of the liver and muscles, but has no definite effect on the liver-fat.

"Time curves" are given showing the simultaneous changes observed during a period of 25 hours after the injection.

As a first phase, corresponding roughly to the first 6 hours after injection, a marked hyperglycaemia occurs, and both liver- and muscle-glycogen show a decrease.

There follows a second phase, which is not completed until about 25 hours after the injection. During this period, the blood-sugar remains at a normal or somewhat low level. The liver-glycogen attains a very high value about the 16th hour and then falls rapidly to the normal. In carbohydrate-fed rats the increase in liver-glycogen is accompanied by an increase in muscle-glycogen, and the subsequent decrease in liver-glycogen by a corresponding decrease in muscle-glycogen. In non-carbohydrate-fed animals the muscle-glycogen changes are retarded.

It is suggested that the changes occurring during the first phase constitute the true adrenaline effect, whereas those of the second phase, being essentially in the opposite direction, are probably secondary effects arising from the disturbance in the endocrine balance produced by the injected adrenaline.

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XXVII. VARIATIONS IN THE CONCENTRATIONS OF MAGNESIUM, CALCIUM AND INORGANIC PHOSPHORUS IN THE SERUM OF THE RABBIT.

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In the intensive study of the behaviour and functions of calcium which has been in progress of recent years there has been a tendency to minimise or overlook any possible rôle played by magnesium, the other generally distributed bivalent cation of the body. For this attitude there has been some excuse. Unlike calcium, magnesium has not been found to vary constantly in any pathological condition, nor, until very recently, has it been influenced by any experimental procedure. Evidence is, however, slowly accumulating of the importance of magnesium in physiological processes and its neglect is no longer justifiable, particularly in the light of the striking effects of magnesium deprivation described by Kruse, Orent and McCollum [1932], and of the results of Greenberg and Mackey [1932, 2], who have shown that after the injection of parathyroid extract a rise in the serum-magnesium occurs even earlier than the well-known rise in the serum-calcium.

The lack of information concerning the factors controlling the level of serum-magnesium led the writer to begin a study of this problem some three years ago and some of the results are presented in this paper. In these studies the rabbit was used as the experimental animal. Unlike those of most of the larger animals used for experimental purposes, certain of the mineral constituents of the rabbit's serum appear to fluctuate under normal conditions within quite wide limits. This is certainly true of the serum-calcium which may vary from 10 to 20 mg. per 100 cc. These variations have been related, at least in part, to dietetic influences. Thus Culhane [1927; 1930] has shown that feeding with cabbage increases the calcium content of the rabbit's serum, while Dupré and Semeonoff [1931] and Bourne and Campbell [1932] confirmed this finding and further demonstrated that the rise in the serum-calcium level consequent on feeding with cabbage was accompanied by a depression in the content of serum-inorganic phosphorus. They found, moreover, that feeding with oats reversed the effects of feeding with cabbage in that the calcium value was depressed while the inorganic phosphorus value was raised. Varying the diet, therefore, provides a convenient method of influencing the calcium and inorganic phosphorus levels of the serum and of studying their relations one to the other under approximately normal conditions. The writer has applied this method to the study of the serum-magnesium and by so doing has been able to demonstrate that there is a definite relationship between the concentrations of calcium and magnesium in the serum.

Experimental.

Calcium, magnesium and, in many cases, inorganic phosphorus determinations have been made at intervals on the serum of rabbits kept under varying dietetic conditions.

Cabbage (outer leaves) and oats were the foodstuffs used and rations were given at 10.30 a.m. and 3.30 p.m. daily.

The experiments fall into three main groups.

I. Animals fed on a mixed diet of oats and cabbage were bled at random, the blood samples being of varying volume and taken at irregular intervals without reference to the time or nature of the previous meal.

II. Rabbits receiving a diet similar to that of the first group were bled every 2nd or 3rd day at 10 a.m., having fasted during the previous night. The usual two meals were given at 10.30 a.m. and 3.30 p.m., and at 4 p.m. the animals were bled again. Values were thus obtained for the sera of animals on a mixed diet in the fasting and absorptive states.

III. One rabbit was fed exclusively on oats for 8 days and then on cabbage for a similar period. A second rabbit was fed exclusively on oats for the whole 16 days. Both animals were allowed water in a separate container during the period of oats feeding. The procedure adopted in taking blood samples was the same as for the animals in Group II, values in the fasting and absorptive states being obtained.

Methods.

The serum obtained from the clotting of blood drawn from the ear was analysed for its calcium content by a modification of the method of Kramer and Tisdall [1921] to be described later. Magnesium was estimated by a slight modification of the method of Cohen [1927] on the supernatant fluid after removal of the calcium. The method consists in the application of the technique of Briggs [1922] for the colorimetric estimation of phosphorus to the magnesium precipitated as magnesium ammonium phosphate. By using N H_2SO_4 for dissolving the washed magnesium ammonium phosphate in place of the $N/100$ H_2SO_4 recommended by Cohen, a considerably more intense colour development is achieved in accordance with the findings of Roe, Irish and Boyd [1926], and it is possible in this way to obtain accurate estimations of calcium and magnesium on 1 cc. of serum in place of the 2 cc. used by Cohen.

Inorganic phosphorus was estimated in 1 cc. of serum by the method of Briggs [1922].

Results.

1. *The effects of allowing serum to stand in contact with the clot.* It was recognised that while serum was in process of separating from the clot diffusion of calcium and magnesium between serum and corpuscles might occur. That red cells contain little or no calcium is a well-established fact. On the other hand, the figures given by Abderhalden [1898], Kramer and Tisdall [1922] and Greenberg and Mackey [1932, 1] all indicate that magnesium is present in rather greater amounts in the corpuscles than in the serum. While it has been shown by Greenberg and Gunther [1930] that the serum-calcium values are uninfluenced by the time serum has stood with the clot and by Greville [1931] that calcium injected intravenously as the laevulate does not enter the corpuscles, no data appear to be available as to whether magnesium diffuses readily from the corpuscles. The matter was therefore investigated. About 60 cc. of human blood were withdrawn and the clot maintained at 37°, all the available serum being poured off

at intervals. Calcium and magnesium estimations were performed in duplicate on the samples so obtained and the results are recorded in Table I.

Table I. *Influence of allowing serum to stand in contact with clot on the calcium and magnesium values of the serum.*

Time after bleeding hours	Volume of serum cc.	Calcium mg./100 cc.	Magnesium mg./100 cc.
1	8	10·83	2·010
		10·72	1·963
1½	11	10·86	1·930
		10·86	1·951
2	4	10·48	2·010
		10·48	2·035
4	4	10·74	2·135
		10·74	2·135
24	1·5	10·95	2·512

From the figures in Table I, it will be seen that the variations in the serum-calcium are quite insignificant while an appreciable rise in the serum-magnesium only occurs after more than 4 hours' standing. Accordingly throughout this work, the serum was poured off after 1 hour's standing at 37° and centrifuged.

2. *The inverse relationship between the concentrations of magnesium and calcium in serum.* There are presented in Table II the results of five serial experiments. These show the rises and falls in the concentrations of calcium and magnesium in the serum of rabbits maintained on various diets, together with the records of the weights of the animals and haemoglobin values at different stages of the experiments.

Table II. *Serum-calcium and serum-magnesium values.*

Day of exp.	Calcium mg./100 cc.	Magnesium mg./100 cc.	Haemoglobin % initial	Weight g.	Blood taken cc.
	Mixed diet. Rabbit 14, bled at random (see Fig. 1).				
0	15·40	1·975	100	2670	3
1	12·71	2·617	100	—	3
2	13·47	2·458	100	—	3
4	10·38	2·796	100	—	3
67	12·88	2·386	90	2010	6
68	12·78	1·746	75	1995	3
70	12·31	2·797	75	—	3
71	11·83	3·768	85	—	3
75	12·88	2·782	75	1840	5
77	13·18	2·255	85	1840	10
81	11·73	2·771	60	—	8
83	13·26	2·296	70	—	12
84	11·74	2·771	55	—	13
	Mixed diet. Rabbit 15, bled at random (see Fig. 2).				
0	15·13	2·094	100	1800	3
1	14·26	2·418	100	—	13
2	—	2·548	90	—	3
4	12·32	2·872	75	—	3
11	11·45	3·950	90	1700	15
12	12·78	3·114	90	—	3
13	14·14	2·014	60	—	3
15	—	—	70	—	3
20	15·66	2·789	90	1710	17
22	15·04	2·207	70	—	3
26	12·32	3·626	90	1610	13

Table II (*cont.*).

Day of exp.	Calcium mg./100 cc.	Magnesium mg./100 cc.	Haemoglobin % initial	Weight g.
Mixed diet. Rabbit 20. Bled 3 cc. morning (m.) (fasting) and afternoon (a.) (absorptive) of alternate days (see Figs. 3 and 4).				
0 m.	14·30	2·323	100	2160
a.	14·44	2·547	75	—
2 m.	12·82	—	90	2010
a.	14·30	—	80	—
4 m.	12·73	2·915	70	2040
a.	13·08	3·793	75	—
7 m.	14·02	2·191	75	2050
a.	14·48	1·816	70	—
9 m.	12·73	2·389	70	1940
a.	13·08	2·911	65	—
11 m.	12·38	2·423	70	2050
a.	14·02	1·896	65	—
14 m.	12·61	2·323	65	1950
a.	14·91	1·866	65	—
16 m.	12·82	2·323	65	—
Diet of oats and water. Rabbit 20. Bled 5 cc. morning (m.) (fasting) and afternoon (a.) (absorptive) of alternate days (see Figs. 5 and 6).				
0 m.	11·40	3·175	100	2010
a.	12·84	3·294	—	—
2 m.	11·40	3·462	—	2070
a.	11·29	3·809	—	—
4 m.	11·73	3·037	70	2080
a.	11·95	3·010	—	—
7 m.	11·51	2·840	—	2020
a.	12·28	2·899	—	—
9 m.	11·40	1·980	70	1910
a.	11·40	2·149	—	—
11 m.	11·51	1·854	—	1870
a.	12·06	1·946	—	—
14 m.	11·73	1·780	60	1780
a.	11·95	2·129	—	—
16 m.	11·51	2·233	—	1720
a.	10·95	1·802	—	—
Diet of oats and water, changed on the 8th day to diet of cabbage. Rabbit 21. Bled 5 cc. morning (m.) (fasting) and afternoon (a.) (absorptive) of alternate days (see Figs. 7 and 8).				
0 m.	12·20	2·465	100	1900
a.	12·31	2·558	—	—
2 m.	10·97	2·666	—	1860
a.	10·20	2·840	—	—
4 m.	11·98	2·389	75	1800
a.	12·09	2·924	—	—
7 m.	11·87	2·324	—	1850
a.	12·31	2·332	—	—
8	Cabbage substituted for oats.			
9 m.	12·20	1·974	70	1800
a.	14·27	1·721	—	—
11 m.	12·86	2·109	—	1790
a.	14·71	2·009	—	—
14 m.	12·53	1·524	70	1720
a.	13·73	1·674	—	—
16 m.	12·31	1·923	—	1700
a.	15·44	2·122	—	—

The significance of these results is readily seen from the series of curves presented in Figs. 1–8. Figs. 1 and 2 represent the results from the sera of two different animals fed on a mixed diet and bled at random times. The remaining curves fall into pairs, *i.e.* Figs. 3 and 4, 5 and 6, 7 and 8 represent the same animal in the fasting and absorptive state.

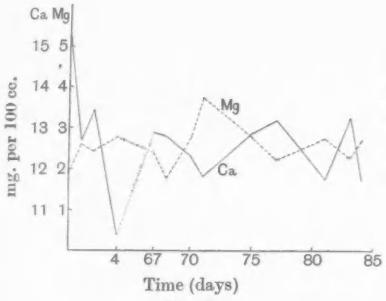

Fig. 1.

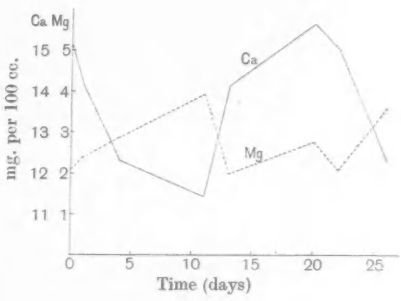

Fig. 2.

Fig. 1. Calcium and magnesium. Rabbit 14, fed on mixed diet; blood drawn at random times.

Fig. 2. Calcium and magnesium. Rabbit 16, fed on mixed diet; blood drawn at random times.

The existence of a definite relationship between the amounts of calcium and magnesium present in the sera is clearly seen in Figs. 1–8. A rise in the amount of calcium is almost invariably accompanied by a fall in the amount of magnesium, and *vice versa*. The relationship is most marked when the rabbits have been fed on a mixed diet (Figs. 1–4) and while the animal is in the absorptive state (Figs. 3, 5, 7). In the same individual in the fasting condition, the relationship although still discernible is less marked (Figs. 4, 6, 8).

3. *The effects of foodstuffs.* Figs. 7 and 8, illustrating an experiment where an animal was fed on oats only and then on cabbage only, suggest that a low calcium and a high magnesium value are associated with feeding oats and a high calcium and low magnesium value with feeding cabbage. A further experiment, lending support to this finding is illustrated in Table III.

Table III. *The effects on the serum-calcium and serum-magnesium of feeding two rabbits* (a) *on the same diet,* (b) *on different diets. Animals bled together at the same intervals after feeding.*

	Rabbit 19		Rabbit 21	
	Calcium mg./100 cc.	Magnesium mg./100 cc.	Calcium mg./100 cc.	Magnesium mg./100 cc.
(*a*)	After oats and cabbage.			
	15·28	1·722	15·73	1·895
(*b*)	After cabbage only.		After oats only.	
	14·81	1·629	12·76	3·634

In this experiment two rabbits were fed on the same rations of oats and cabbage simultaneously. One hour later they were bled in quick succession. The calcium and magnesium values so obtained may be regarded as virtually those

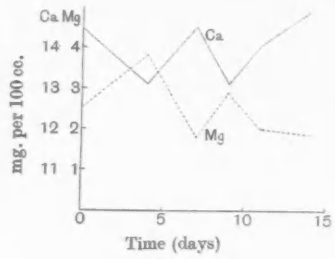

Fig. 3.

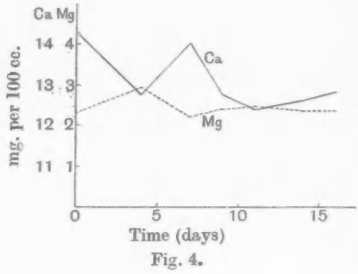

Fig. 4.

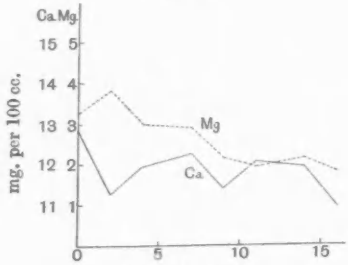

Fig. 5.

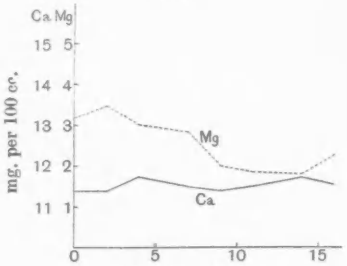

Fig. 6.

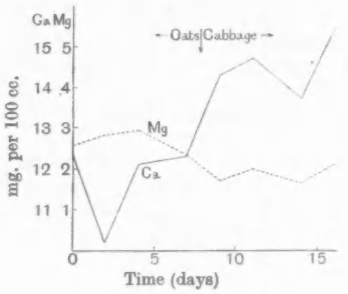

Fig. 7.

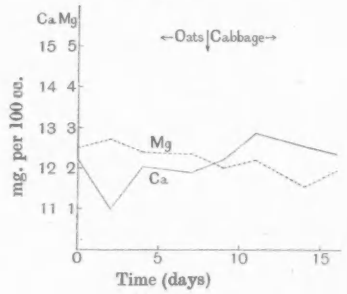

Fig. 8.

Fig. 3. Calcium and magnesium. Rabbit 20, fed on mixed diet; afternoon (absorptive) values.

Fig. 4. Calcium and magnesium. Rabbit 20, fed on mixed diet; morning (fasting) values.

Fig. 5. Calcium and magnesium. Rabbit 20, fed on oats; afternoon (absorptive) values.

Fig. 6. Calcium and magnesium. Rabbit 20, fed on oats; morning (fasting) values.

Fig. 7. Calcium and magnesium. Rabbit 21, fed on oats, then cabbage; afternoon (absorptive) values.

Fig. 8. Calcium and magnesium. Rabbit 21, fed on oats, then cabbage morning (fasting) values.

associated with cabbage feeding, since rabbits offered cabbage and oats invariably consumed the former first. The calcium values are relatively high, while those for magnesium are low. It will be observed that the values obtained for the two animals correspond very closely, and it may be noted at this point that it has been repeatedly found that animals, similarly fed, give values for calcium and magnesium which lie very close together. One of the rabbits was then placed on a diet of oats, the other on one of cabbage. Again the animals were bled at the same interval after feeding, and the values obtained indicate that feeding with cabbage exclusively has effected little change in either calcium or magnesium values, but that a marked effect has been elicited by feeding with oats. The magnesium value has risen to a high level in the rabbit fed on oats, while the calcium value has fallen.

4. *The relationship between the amounts of the serum-calcium and serum-inorganic phosphorus.* Typical of the results obtained when estimations of inorganic phosphorus in addition to those of calcium and magnesium were carried out are the figures presented in Table IV.

Table IV. *Serum-calcium, serum-magnesium and serum-inorganic phosphorus values on mixed diet.*

Day of exp.	Calcium mg./100 cc.	Magnesium mg./100 cc.	Inorganic phosphorus mg./100 cc.	Haemoglobin % initial	Weight g.
Rabbit 19. Bled 5 cc. morning (m.) (fasting) and afternoon (a.) (absorptive) of alternate days.					
0 m.	15·28	2·163	2·100	100	2700
a.	15·39	2·389	1·768	80	—
2 m.	15·16	—	5·626	90	2660
a.	14·34	—	3·904	85	—
4 m.	11·89	2·122	5·626	80	2660
a.	10·95	3·877	4·826	75	—
7 m.	13·17	2·340	4·083	80	2570
a.	14·11	2·109	3·025	75	—
9 m.	12·71	2·422	4·894	70	2500
a.	10·95	3·119	5·669	60	—
11 m.	12·00	2·457	5·669	70	2570
a.	13·29	1·795	2·940	70	—
14 m.	13·06	1·748	4·083	70	2600
a.	13·99	1·382	2·647	60	—
16 m.	13·17	2·249	5·316	75	—

If a curve be drawn showing the rises and falls in the figures for calcium and inorganic phosphorus, it becomes at once apparent that these two bear a reciprocal relationship to one another as observed by a number of workers [Bourne and Campbell, 1932; Dupré and Semeonoff, 1931]. Since the calcium and magnesium contents are also in reciprocal relationship, it follows that the amount of magnesium tends to rise and fall in the same direction as does the inorganic phosphorus and in the direction opposite to that of the calcium.

5. *Variations in the rabbit's serum-magnesium under normal conditions.* While there have been many extended observations of the amounts of calcium in the rabbit's serum, very few data are available as to the amount of magnesium normally present. The magnesium ranges encountered in Tables II and IV representing series of observations on four animals in the fasting and absorptive state, are summarised in Table V. Calcium values are given for the purpose of comparison. It will be seen that variations occur in the serum-magnesium of 1·4 to 3·9 mg. per 100 cc. and also that in general the range of variation is greater when the animal has been feeding than when it is in the fasting condition.

Table V. *Ranges of variation of serum-calcium and serum-magnesium under normal conditions.*

	Calcium mg./100 cc.		Magnesium mg./100 cc.	
Table no.	Fasting	Absorptive	Fasting	Absorptive
II Figs. 3, 4	12·4–14·3	13·1–14·9	2·2–2·9	1·8–3·8
II Figs. 5, 6	11·4–11·7	11·0–12·8	1·8–3·5	1·8–3·8
II Figs. 7, 8	11·0–12·9	10·2–15·4	1·5–2·7	1·7–2·9
IV	11·9–15·3	11·0–15·4	1·7–2·5	1·4–3·9

Discussion.

In endeavouring to account for the effects on the calcium and inorganic phosphorus content of rabbit's serum of feeding cabbage and oats respectively, Bourne and Campbell [1932] suggested that the explanation might lie in the close parallelism existing between the relative amounts of these constituents in the foodstuff and in the serum after the foodstuff had been absorbed. Thus, in their experiments, when cabbage, which is rich in calcium and poor in phosphorus, was fed the serum-calcium value was raised while the serum-inorganic phosphorus value fell. On the other hand, feeding with oats, which is rich in phosphorus but poor in calcium, led to a rise in the serum-inorganic phosphorus and a fall in the serum-calcium. A similar parallelism can be traced between the amounts of calcium and magnesium in cabbage and oats respectively and the calcium and magnesium values of the serum during the absorption of each foodstuff. The calcium and magnesium contents of oats and cabbage as estimated by various authorities are given in Table VI.

Table VI. *The calcium, magnesium and phosphorus contents of oats and cabbage (percentages of fresh foodstuff).*

Oats			Cabbage			
Calcium	Magnesium	Phosphorus	Calcium	Magnesium	Phosphorus	Authority
0·080	0·134	0·349	0·406	0·055	0·035	(1) König [1904]
—	—	—	0·072	0·023	0·058	(2)
0·117	0·181	0·364	—	—	—	Greaves and Hirst [1929]
—	—	—	0·053	—	0·028	(3) Peterson and Peterson [1926]
—	—	—	0·045	0·015	0·029	(3) Sherman [1928]
0·077	—	—	0·161	—	—	(1) Bourne and Campbell [1932]
—	—	—	0·476 to 1·058	—	—	(1) Cowell [1932]
—	—	—	0·026 to 0·034	—	—	(2)

(1) Outer leaves. (2) Heart. (3) Stripped of outer leaves.

Feeding with cabbage, which is rich in calcium but poor in magnesium, leads to a rise in the serum-calcium and a fall in the serum-magnesium: feeding with oats, which is poor in calcium but rich in magnesium, depresses the serum-calcium and raises the serum-magnesium value, at any rate initially. In considering the absorption of calcium and magnesium from the alimentary canal, the amount of phosphorus present in the foodstuff has to be taken into account: where the phosphorus is low, calcium and magnesium will be readily absorbed, where it is high, the excess of phosphate in the bowel will combine with the calcium and magnesium to form relatively insoluble phosphates which enter the blood-stream with difficulty. It seems reasonable to suppose that herein lies the

explanation of the lowering of the magnesium as well as of the calcium value when feeding with oats, with its high phosphorus content, is persisted in (Figs. 5 and 6).

To suggest, however, that the inverse relationship existing between the serum-calcium and serum-magnesium is due solely to the relative amounts of these elements in the diet, would hardly suffice to account for the delicate way in which their levels are adjusted. The conception of balanced systems of ions, introduced by Ringer [1882] and developed by Loeb [1900] and many later workers, is universally recognised as being of the first importance in all manifestations of physiological activity and probably has an application to the present problem. Ions may, under different circumstances, act in synergism or antagonism. Magnesium and calcium provide an outstanding example of ion antagonism in connection with magnesium narcosis. Meltzer and Auer [1908] showed that an animal, anaesthetised by the injection of magnesium sulphate, could be almost instantly restored to normal activity by the subsequent injection of a calcium salt. Here, however, large excesses of either ion are in view, conditions different from those now being considered. In certain circumstances magnesium has been shown to exert an action similar to that of calcium and opposite in character to those of the univalent cations, sodium and potassium. Thus Lillie [1911], working with star-fish and sea urchin eggs, showed that while sodium increased the permeability of the cell membrane, calcium and magnesium had the reverse effect. Again, the bivalent and univalent cations have diametrically opposite effects on the cell protoplasm, as shown by the results of Chambers and Reznikoff [1925], who found that on injection into amoebae, sodium and potassium chlorides caused liquefaction of the protoplasm at the site of injection, while calcium and magnesium chlorides behaved in a reverse manner and caused solidification.

It seems, therefore, possible that the inverse relationship of the calcium and magnesium of serum reflects a mechanism whereby the total bivalent cation of the blood is kept at a relatively constant level. On such an assumption the wide range of individual variation of the two metals is no longer so surprising and brings the experimental findings on the variations of calcium and magnesium in the blood of the rabbit into line with man and other species in which the levels of the serum-calcium and other mineral constituents are much more rigidly defined. The apparent need for greater latitude in the case of the rabbit may conceivably be related to the more variable mineral content of a herbivore's diet.

Summary.

1. An inverse relationship between the concentrations of calcium and magnesium in the serum of the rabbit has been described. This is particularly prominent when a mixed diet is being given and where blood samples are taken with the animal in the absorptive state.

2. The concentrations of both calcium and magnesium in the serum of the rabbit are influenced by the character of the diet.

3. The existence of an inverse relationship between calcium and inorganic phosphorus in the serum, as described by previous workers, has been confirmed.

4. Consequently, the level of serum-magnesium, while moving in the opposite direction to that of calcium, moves in the same direction as that of inorganic phosphorus.

5. No diffusion of magnesium from the red cell into the serum occurs at 37° unless the time of standing be greater than 4 hours.

The writer wishes gratefully to acknowledge the help and encouragement given to him by Prof. H. J. Channon during the progress of this research. His thanks are also due to Prof. W. Ramsden at whose instigation it was originally undertaken and to the British Medical Association for the award of a Research Scholarship.

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XXVIII. THE BIOCHEMICAL OXYGEN DEMAND OF SEWAGE.

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When crude sewage is run into a river its constituents usually undergo oxidation until simpler stable end-products are produced. The oxidation occurs largely at the expense of dissolved oxygen in the river water and, in certain circumstances, may lead to such serious depletion of the dissolved oxygen that aquatic life is affected. One of the main objects of sewage purification works is to carry these oxidations to a point where the further effect of the sewage upon the dissolved oxygen supply of the river will be unimportant. The actual mechanism underlying this oxygen demand of sewage has not yet been satisfactorily demonstrated. Various authors have ascribed the uptake of oxygen to the agency of living organisms, such as bacteria and protozoa, others have suggested that the change is largely chemical, whilst most workers consider it to be a combination of the purely chemical process and the biological one. In the present paper it is hoped to bring forward definite experimental evidence to show that the oxygen absorption due to purely chemical, as distinct from biochemical, processes is usually negligible, and that the presence of organisms—whether living or dead—or their enzymes is essential to a large uptake of oxygen.

Experimental.

As a test of the extent to which this process of purification has proceeded, the sewage or sewage effluent is examined by one of various methods, but that which is most generally accepted is known as the Rideal-Stewart modification of Winkler's method. In this test, the sewage to be examined is diluted to a suitable concentration, then saturated with air and poured into two bottles of known capacity; in our experiments we have used bottles of approximately 400 cc. capacity. The sewage is diluted with tap water. The filled bottles are allowed to stand, with occasional tapping, to ensure that bubbles of excess air have time to escape. Well-fitting stoppers are then inserted and one of the bottles is incubated at 22° for 5 days in the dark. As an incubator at 22° was available this temperature was chosen instead of that of 18·3° recommended by the Royal Commission on Sewage Disposal. The dissolved oxygen present in the other bottle is determined at once by the modified Winkler method [Royal Commission on Sewage Disposal, 1913]. After the 5 days' incubation at 22° the dissolved oxygen present in the second bottle is similarly determined. From the difference between the two readings, knowing the capacity of the two bottles and the dilution of the sewage, the 5-day biochemical oxygen demand of that sewage can be expressed numerically. An effluent which has a 5-day oxygen demand of two parts or less per hundred thousand is usually considered satisfactory in those instances where the small volume of the diluting stream renders it necessary to secure a high degree of purification.

In experiments with sterilised sewage, it was obviously necessary to avoid bacterial contamination in putting up the tests. Sterilised glassware was employed throughout and the sewage, suitably diluted, was always sterilised in a vessel approximately twice the capacity of the contained sewage. Prior to filling the 400 cc. bottles, the cotton-wool plug of the flask containing the diluted sewage was replaced by a sterile rubber bung and the whole vigorously shaken for at least 4 minutes. It was then allowed to stand a few minutes and poured into the smaller bottles under a large sterilised funnel. During the filling process the stoppers of the 400 cc. bottles were placed on sterile tiles beneath sterile beakers, whilst the necks of the bottles were covered by sterilised aluminium caps. These caps were only removed immediately before pouring. After filling, the cap was immediately replaced and the bottle tapped and allowed to stand for some minutes to allow excess air bubbles to rise to the surface. The stoppers were then inserted, and the bottle incubated at 22°. When it was necessary to add organisms, enzyme preparations or inhibitors to the sewage, this was done after the shaking, and even distribution of the added product was secured by shaking the sewage for a further short period before filling the individual 400 cc. bottles. The contents of the bottles were examined bacteriologically just before measurement of dissolved oxygen. This usually meant the removal of 1–2 cc. of the contents. Bacterial counts were carried out by two methods. When it was only necessary to ascertain if the bottles were sterile or grossly contaminated, definite quantities of the fluids were placed in sterile Petri dishes to which 15 cc. of melted agar were subsequently added. If, however, accurate counts of the viable organisms were required, the roll tube method [Wilson, 1922] was used; serial dilutions of the sewage were made and inoculated into melted agar in tubes which were rolled and incubated. All plates and tubes were incubated at 22° for 5 days before counting.

In the enzymic examination of the various organisms used, the latter were usually grown on agar for 24–48 hours, washed off with sterile Ringer solution, centrifuged and washed twice, and finally made up to a thick emulsion (about 15×10^{10} organisms per cc.) with this fluid. For the examination of dehydrogenase properties the usual Thunberg methylene blue technique was followed; 1 cc. of organisms (previously diluted if necessary) was used, together with suitable quantities of phosphate buffer (p_H 7·4), the substrate and 1 cc. of 1/5000 methylene blue solution. When crude sewage was used as a substrate in these tests, 4 or 5 cc. were usually employed. All the necessary controls were carried out, *viz.* without substrate, without organisms or with boiled organisms. In examining for oxidase the technique described by Keilin [1929] was followed. To 1 cc. of washed organisms in an open test-tube were added 0·5 cc. of *p*-phenylenediamine solution and 1 cc. of 7·4 phosphate buffer. This was shaken for 10 minutes; the development of a deep purple colour indicated the presence of the indophenol oxidase (provided the control tube without organisms remained colourless). Sometimes, as mentioned by Keilin, it was necessary to inhibit the reducing action of dehydrogenase systems by the addition of urethane before a positive indophenol oxidase reaction was obtained on shaking, as described above.

Comparison of bacterial counts and the oxygen demand of crude sewage.

If a series of six 400 cc. bottles be filled from the same bulk sample of oxygen-saturated diluted sewage and one bottle be examined each day both for viable count of bacteria and for oxygen absorption, results are usually obtained similar to those given in Table I.

Table I. *The relation of viable count of bacteria and oxygen absorption of crude sewage under the conditions of the Rideal-Stewart Test.*

Crude sewage diluted 1 : 20 with tap water. One bottle examined each day for the number of viable organisms present and the amount of dissolved oxygen. The bacterial counts relate to the number of organisms actually present in the bottle per cc. The Biochemical Oxygen Demand figures refer to the B.O.D. of undiluted C.S., the figures giving the amount of oxygen absorbed, in parts per 100,000, from the beginning of the experiment to the day on which the bottle was examined.

Exp.	Days at 22°	0	1	2	3	4	5
1	Viable count	1,209,000	2,883,000	1,555,000	268,700	143,600	286,500
	B.O.D.	—	3·9	5·7	9·0	11·9	13·2
2	Viable count	1,465,000	3,116,000	1,233,000	761,200	179,600	66,700
	B.O.D.	—	3·6	6·5	9·1	12·3	12·6

From these two experiments it is seen that there is no direct correlation between the number of viable bacteria and the oxygen absorption, for the latter rises throughout the 5 days whilst the viable count increases rapidly to a maximum after 1 day's incubation at 22° and then rapidly falls. Although these figures are illustrative of the majority of the experiments we have carried out, yet occasionally we find that both the viable count and the oxygen absorption increase throughout the 5 days' incubation. Sometimes the greater proportion of the total oxygen absorption takes place in the first 2 days. Even so there appears to be no correlation between the number of viable organisms and the amount of oxygen absorbed. This conclusion is further supported by the figures given in Table III.

The oxygen absorption of sterile sewage.

If diluted crude sewage be sterilised, either by autoclaving at 120° for 20 minutes or by filtration through Seitz filters, then it will absorb no oxygen during the ordinary 5-day test, provided that precautions are taken to ensure sterility during the filling of the bottles and throughout the experiment. If, however, an accidental contaminant gains access to the sewage, this is frequently followed by

Table II. *The oxygen absorption of sterile crude sewage as compared with that of crude sewage or sterile crude sewage* plus *organisms.*

C.S. 120° = autoclaved crude sewage.
C.S. sf. = sewage filtered through a Seitz filter.

Sewage preparation	Viable count 0 day	Viable count 5th day	B.O.D. p.p. 10^5
Sewage A series.			
C.S. (diluted 1/20)	260,000	143,000	16·9
C.S. 120° (diluted 1/20)	0	0	0
,, +C.S. seeding	1,500	6,000,000	18·0
,, +sewage organisms	1,500,000	12,000,000	18·0
,, +sewage organisms 120°	0	0	0·6
C.S. 120° (undiluted)	0	0	0·22
C.S. sf.	0	0	0
,, +C.S. seeding	1,500	9,000,000	17·3
,, +sewage organisms	1,500,000	13,000,000	17·8
,, +sewage organisms 120°	0	0	0
Sewage B series.			
C.S. (diluted 1/50)	150,000	460,000	40·0
C.S. 120° (undiluted)	0	0	0·2

an oxygen uptake equal to that of the unsterilised sewage. The addition of a small seeding of crude sewage to the sterile sewage (1 cc. to 2 litres) also results in a large oxygen absorption. That the oxygen uptake due to purely chemical changes is practically negligible is more clearly shown by the fact that crude sewage, if sterilised, will take up little oxygen even when undiluted. Illustrative results are shown in Table II.

As a further test of the importance of bacteria in this process of absorption of oxygen by crude sewage, a suspension of washed bacteria obtained from a mixed culture of organisms grown from sewage was inoculated into the sterilised (autoclaved or filtered) sewage. There followed a large absorption of oxygen, which did not take place, however, if the washed sewage organisms were boiled previous to inoculating the sterile sewage.

Oxygen absorption of sterile sewage inoculated with pure cultures of bacteria.

The experiments described in the previous section indicate that the addition of a mixed culture of living bacteria to sterile sewage will lead to oxygen absorption. It was decided to see whether the introduction of a pure strain of bacteria would similarly lead to oxygen absorption. Suspensions of washed cells of *Pseudomonas fluorescens* and of *Bact. coli* were prepared as described in the experimental section. These suspensions were used so as to reduce to a minimum the possibility of adding oxidisable substrate with the bacteria. It was found that the introduction of a small number of *Ps. fluorescens* cells resulted in rapid growth of the organism and in large oxygen absorption, whilst the inoculation of small numbers of *Bact. coli* led to no appreciable oxygen absorption and no increase in numbers of viable organisms. If, however, the sterile sewage were inoculated heavily (several million per cc.) with *Bact. coli* then large oxygen absorption took place, although here again there was no apparent growth of the organism. The inoculation of sterile tap water with a like amount of *Bact. coli* only resulted in a small oxygen uptake and hence we may conclude that the utilisation of the oxygen was largely due to the oxidation of the sewage constituents owing to the presence of the *Bact. coli* cells. Naturally the amount of oxygen absorbed by the thick bacterial suspension itself will vary with the number of cells present and their enzymic activity, and consequently there is a limit to the number of organisms which it is possible to add to a medium saturated with oxygen and still be able to ascertain, by these methods, whether the cells facilitate the oxidation of that medium. If the thick bacterial suspension be boiled before its addition to the sterile sewage no oxygen absorption takes place. These results are set out in Table III.

Further examination, however, using sewages obtained at different times and apparently differing somewhat in chemical constitution, indicated that whilst the addition of small numbers of *Ps. fluorescens* to any of the sterile sewages invariably resulted in a large oxygen absorption yet small inocula of *Bact. coli* behaved erratically. With some sewages, which enable the *Bact. coli* to grow, oxygen absorption took place, whilst with others, in which the organism did not appear to be able to multiply, oxygen uptake did not result from the inoculation. When a number of other organisms were similarly examined they were found to behave either like *Ps. fluorescens* or *Bact. coli*. *Ps. pyocyaneus*, *Staph. albus* and a number of organisms isolated from sewage were able to proliferate when inoculated in small numbers (about 1000 per cc.) into sterile sewage, effecting a large oxygen absorption, whilst other organisms, such as *Bact. aerogenes*, *Chr. prodigiosum*, *Proteus* and one or two organisms isolated from sewage gave erratic results similar to those obtained with *Bact. coli*.

Table III. *Viable counts and oxygen absorption figures in parts per* 100,000 *of autoclaved sewage to which have been added various preparations of organisms.*

The counts refer to the number of viable bacteria per cc. actually present in the bottle contents whilst the oxygen demand (B.O.D.) figures refer to the undiluted sewage. As the crude sewage has been diluted 1 : 20 the water control B.O.D. figures have been similarly multiplied by 20 for purposes of comparison. In the table *Bact. coli* 100° and *Ps. fluorescens* 100° were obtained by heating the thickest suspension of these organisms to 100° for 30 minutes.

Sewage system		0 day	1 day	2 days	3 days	4 days	5 days
C.S. 120° + C.S. seeding	V.C.	2,400	2,240,000	13,700,000	5,500,000	1,200,000	880,000
	B.O.D.	—	9·1	15·8	16·1	16·4	16·0
C.S. 120° + *Bact. coli*	V.C.	116,250,000	60,800,000	8,000,000	15,700,000	4,400,000	5,800,000
	B.O.D.	—	7·9	8·6	13·3	14·5	15·1
C.S. 120° + *Bact. coli* 100°	V.C.	0	0	0	0	0	0
	B.O.D.	—	0	0	0	0	0
C.S. 120° + *Bact. coli*	V.C.	9,700,000	2,000,000	54,000	6,000	250	1,800
	B.O.D.	—	1·4	2·2	2·4	2·9	3·1
C.S. 120° + *Bact. coli*	V.C.	618,000	439,000	1,000	20	0	0
	B.O.D.	—	0	0	0	0	0·6
Tap water 120° + *Bact. coli*	V.C.	76,000,000	76,000,000	79,900,000	76,000,000	56,000,000	44,000,000
	B.O.D.	—	0·8	1·4	1·8	2·4	3·4
C.S. 120° + *Ps. fluorescens*	V.C.	58,000,000	30,000,000	15,800,000	10,900,000	5,300,000	8,900,000
	B.O.D.	—	15·8	15·8	15·9	15·8	16·0
C.S. 120° + *Ps. fluorescens* 100°	V.C.	0	0	0	0	0	0
	B.O.D.	—	0	0	0	0·2	0·4
C.S. 120° + *Ps. fluorescens*	V.C.	3,300	260	540,000	15,000,000	19,000,000	50,000,000
	B.O.D.	—	0·8	1·3	11·8	13·8	15·5

Some preliminary enzyme observations.

An enzymic study of these organisms showed that all those organisms which invariably induced oxygen absorption possessed active dehydrogenase systems for all the sewages, whilst those organisms which behaved erratically with different sewages frequently showed no sewage-dehydrogenase powers with those sewages upon which they appeared unable to grow. Furthermore the oxidase activity of the *Ps. fluorescens* group was invariably strongly marked, whilst the activity of this enzymic system appeared to be either absent from, or very weakly present in, the *Bact. coli* group of organisms.

Inhibition experiments. The presence of *M*/1000 KCN or of 5 % urethane in any enzyme system will respectively completely inhibit the activity of Keilin's indophenol oxidase or of dehydrogenases. Crude sewage, saturated with air, was incubated at 22° in the usual way in the presence of one or other of these inhibitors, and it will be seen from Table IV that the inhibition of oxygen uptake is complete in each instance, whilst a smaller concentration of KCN, *viz.* *M*/10,000, did not inhibit the reaction.

Table IV. *The effect of the addition of KCN or of urethane upon the oxygen absorption by crude sewage.*

No actual counts of viable bacteria were made but the sign + indicates that the colonies were uncountable when 0·01 cc. of the diluted sewage preparation was inoculated on to an agar plate. When a negative result was obtained it is recorded by a zero sign.

Experiment		0 day	1	2	3	4	5
1. Crude sewage alone	B.O.D.	—	9·6	14·3	16·2	16·0	16·1
	Viable bacteria	+	+	+	+	+	+
2. Crude sewage + *M*/10,000 KCN	B.O.D.	—	9·0	14·0	16·0	16·1	15·9
	Viable bacteria	+	+	+	+	+	+
3. Crude sewage + *M*/1000 KCN	B.O.D.	—	0	0	0	0	0·2
	Viable bacteria	+	+	+	+	+	+
4. Crude sewage + 5 % urethane	B.O.D.	—	—	0·4	—	—	1·4
	Viable bacteria	4000	—	0	—	—	0

In the urethane experiments the sewage was rendered sterile and so the deduction that dehydrogenases are essential to the oxygen absorption cannot be rigidly upheld but the sewage with *M*/1000 KCN still contained a large number of viable organisms and hence it is probable that oxidase activity is necessary for the oxygen uptake.

Experiments with treated organisms. In order to demonstrate more clearly that viable organisms are not essential to the oxygen absorption provided the necessary enzymes are present, suspensions of *Ps. fluorescens* were treated in various ways in the hope of destroying their power of proliferation but of leaving their enzymes still active. Owing to the fact that complete sterility was essential this task was found to be extremely difficult, for usually sufficient viable cells were left by most treatments to invalidate the experiments, or else, with complete sterility, the enzymes themselves were either destroyed or their activities rendered extremely feeble. Finally, success was achieved by treatment of the bacterial suspension with formaldehyde. In this treatment a bacterial suspension (*e.g.* 10 cc.) was submitted to the action of formaldehyde, say 0·5 %, for some hours. It was then centrifuged and the deposit made up in Ringer solution to 10 cc. When this is diluted to 1500 cc. by addition to the sewage the concentration of aldehyde is negligible. The effect of this treatment varied with the concentration both of the cells and of the aldehyde, and also with the temperature and time of treatment so that for each suspension it was essential to carry out a series of tests in order to find out the conditions of treatment necessary to render the suspension sterile whilst leaving the enzymes active. By careful work, however, this result can be achieved and Table V sets out an experiment (one of several) whereby we secured a good oxygen absorption by sterile sewage on the addition of aldehyde-treated *Ps. fluorescens*. At no time during the experiments could living cells be demonstrated. These concentrations of aldehyde were tested in the experiments cited and they go to show that the greatest concentration (here 1 %) of aldehyde not only sterilised the organism but also destroyed the dehydrogenases and considerably weakened the oxidase enzymes. 0·5 % formaldehyde however left these enzymes active whilst rendering the suspensions sterile, whilst 0·2 % formaldehyde neither inactivated the enzymes nor sterilised the suspension. A vigorous uptake of oxygen was obtained when the 0·2 % or the 0·5 % aldehyde-treated organism was inoculated into either sterile crude sewage or into some other sterile medium, such as glucose, lactate or formate solution. From the table it is seen that where oxygen absorption took

place the treated organism possessed active oxidase and dehydrogenase systems, although unfortunately in these experiments no tests were made for the sewage-dehydrogenase. Dehydrogenases for the other substrates were shown to be present whenever the treated organism resulted in oxidation of that substrate.

Table V. *Oxygen absorption by sterile formaldehyde-treated suspensions of* Ps. fluorescens *in the presence of sterile sewage or other substrate adjusted to* p_H *7·2.*

10 cc. of a thick suspension of treated organisms were added to 1400 cc. of sterile medium saturated with air. Suspensions were treated for 20 hours at room temperature with the formaldehyde.

It should be remembered that the observed oxygen absorption in the Rideal-Stewart method is limited by the saturation value of the oxygen and hence, to discover how much oxygen a substrate will utilise it is necessary to choose a dilution of the substrate so that the available oxygen will be in excess. Thus a dilution of 1 : 20 was chosen below and the oxygen demand of the C.S. obtained by multiplying the observed figure by 20. In order to bring the B.O.D. figures for the undiluted sewage and other substrates into line with these observations, they also have been multiplied by 20. The sign + in the columns for viable bacteria means that bacterial growth was obtained when 1 cc. of the mixture in the bottles was grown by the agar plate method. 0 means that no growth was obtained in 7 days.

Concentration of H.CHO used in treatment of organisms %	Substrate	Viable bacteria: 0 day	Viable bacteria: 3rd day	Viable bacteria: 5th day	B.O.D.: 3 days	B.O.D.: 5 days	Oxidase	Dehydrogenase
0·2	Crude sewage 120° (undiluted)	+	+	+	15·2	15·5	+	—
	Crude sewage 120° (1 : 20)	+	+	+	13·2	14·8	+	—
	Lactate *M*/1000	+	+	+	13·2	14·3	+	+
	Formate *M*/1000	+	+	+	14·3	14·5	+	+
	Glucose *M*/1000	+	+	+	12·8	13·9	+	+
0·5	Crude sewage 120° (undiluted)	0	0	0	10·3	12·4	+	—
	Crude sewage 120° (1 : 20)	0	0	0	9·4	14·9	+	—
	Lactate *M*/1000	0	0	0	10·0	12·6	+	+
	Formate *M*/1000	0	0	0	9·9	12·0	+	+
	Glucose *M*/1000	0	0	0	13·2	13·3	+	+
1	Crude sewage 120° (undiluted)	0	0	0	0	0	(+)	—
	Crude sewage 120° (1 : 20)	0	0	0	0	0	(+)	—
	Lactate *M*/1000	0	0	0	0	0	(+)	0
	Formate *M*/1000	0	0	0	0	0	(+)	0
	Glucose *M*/1000	0	0	0	0	0	(+)	0

Discussion.

It would appear from the foregoing experiments, as has been frequently suggested, that the oxygen absorption of sewage is dependent largely upon the presence of micro-organisms. Further it is shown that oxygen absorption takes place in the presence of enzymically active dead cells or cell débris. That the oxidation of sewage constituents is negligible in the absence of these biological catalysts has been demonstrated by showing that crude sewage, sterilised by heat or by filtration through bacteriological filters, does not absorb oxygen. Even undiluted crude sewage, which when unsterilised gives a 5-day figure of 40 parts per 10^5, scarcely effects any oxygen absorption after being sterilised by heat or

filtration. Such reagents as toluene, chloroform, ether, *etc.*, if in a concentration sufficient to assure sterility, similarly completely inhibit oxygen absorption by crude sewage, presumably because they inhibit enzyme action (Table VI).

Table VI.

In the following experiments crude sewage (1 : 40) was allowed to stand for 2 hours with the disinfectant given below. It was then saturated with oxygen, by shaking, and poured into bottles. The oxygen absorption was measured by the usual method. Control bottles with the disinfectant made up in tap-water were also put up and the results given are the differences between the controls and the corresponding sewage experiments. Bacterial growth was estimated by plate inoculation.

Disinfectant	Bacterial growth 0 day	Bacterial growth 3rd day	O_2 absorbed 3 days	Bacterial growth 5th day	O_2 absorbed 5 days
—	+ + +	+ +	27·2	+ +	35·3
2 % $CHCl_3$	0	0	0	0	0
5 % „	0	0	0	0	0
2 % Toluene, 37°	0	0	0	0	0·6
5 % „	0	0	0	0	0
5 % Ether	0	0	0	0	0
10 % „	0	0	0	0	0

Although it may be suggested that autoclaving crude sewage may result in a change in its chemical constitution, yet it is unlikely that the readily oxidisable constituents will also be changed or removed by filtration through a Seitz filter, or by the addition of such substances as toluene, chloroform and ether. Again, if small amounts of crude sewage or a seeding of a particular organism be added to a sterilised sewage then the oxygen absorption is as large as it was previous to sterilisation. Generally speaking then we may conclude that a direct chemical oxidation is rare in crude domestic sewage. Certain industrial effluents may lead to a large chemical oxidation, depending upon the chemical nature of the substances in such effluents. In this connection we have been unable to demonstrate the presence of compounds containing the sulphydryl group in the sewages we have examined, but we have, on several occasions, been able to induce the formation of these substances by storing the crude sewage in well-filled stoppered bottles (that is, anaerobically) for some days. A positive nitroprusside reaction is then obtained which is, at least in part, due to hydrogen sulphide.

It has been shown that the addition of bacterial cells, in pure or mixed suspensions, is capable of setting up a large oxygen absorption by sterilised sewage. Preliminary experiments, using a bacteria-free suspension of a protozoon (*Polytoma uvella*) in pure culture, failed to lead to any oxygen absorption although there were live protozoa present at the end of the 5-day period. This negative result may be due to the comparatively small numbers of protozoa added, although it may be mentioned that in most sewages, compared with the number of available bacteria, protozoa are indeed few. It is probable that bacteria form the most important part of a catalytic system which is essential for sewage oxidation. This oxidation by bacteria or their enzymes may be accompanied by bacterial multiplication, but there appears to be no direct correlation between the increase in the number of living bacteria and the oxygen absorption, although it is admitted that deductions from figures which ignore the rate of death of the organisms must not be considered too seriously. This same objection applies to the conclusion that living but non-proliferating bacteria can induce sewage oxidation, this conclusion being drawn from the experiments where large numbers of organisms were added to sterile sewage resulting in a good oxygen absorption but no apparent cell growth. The fact that certain bacterial enzymes

are necessary to the mechanism of oxygen absorption appears to be demonstrated by the inhibition experiments, for *M*/1000 KCN, which inhibits the indophenol oxidase, and 5 % urethane, which inhibits dehydrogenases, each largely arrests the oxygen absorption by non-sterile sewage. These results are not due to sterility, for KCN, in this concentration, does not sterilise the sewage, although 5 % urethane does. That bacterial enzymes can induce oxygen absorption by sterile sewage is proved by the experiments with the formaldehyde-treated organisms. In these experiments the bacterial suspension was first treated with the aldehyde so that it was completely sterile but retained its oxidase and dehydrogenase mechanisms in a state of marked activity. These experiments then indicate the importance of the dead cell and enzymically active material of sewage and sewage sludge in the purification processes of sewage.

Naturally it is not suggested that the enzymic systems still active in dead bacteria are more important, quanta for quanta, than those possessed by living and multiplying bacterial cells. By far the greater proportion of the bacteria in sewage are, however, dead as judged by the fact that they are incapable of multiplication. The proportion of viable to total bacteria in samples of the sewage effluents with which we have worked is shown in Table VII.

Table VII.

Total	Viable	T./V.
1,207,800,000	3,229,200	374·0
690,070,000	4,500,000	172·5
1,017,800,000	3,813,400	266·9
4,017,900,000	4,404,876	911·5
3,614,100,000	2,918,678	1238·2

In the aggregate, then, the enzymic action of the dead cells is probably far greater than that of the living. The exact part played by these oxidising enzymes in the processes of sewage purification cannot be decided with the present technique. The problem is now being attacked, using other methods.

From the above experiments it follows that the 5-day test is dependent upon two main variables. First, the chemical nature of the sewage and second, its enzymic content (associated with living or dead cells). These factors are not independent variables but are intimately interconnected, for the first must supply the substrates to the second. As river waters may contain varying substances in solution and a variable bacteriological flora, one ought, when deciding whether an effluent will have any harmful effect upon the dissolved oxygen value of a river, to dilute the effluent with the river water. In this way the possibility of the effluent supplying one of the above variables and the river the other is allowed for. Generally speaking, however, the likelihood of the enzymes and substrates being supplied independently by the effluent and the river will be small but it is a possibility that should not be entirely forgotten, especially where several trade wastes may gain access to a river in the same locality. The effective oxygen absorption that will arise from a trade effluent may also be missed if the effluent contains some substance, *e.g.* acid, which inhibits the bacteriological enzymes. When the effluent gains access to the river the inhibitor may be so diluted that it will not affect the river flora and oxygen-absorption will ensue. It is possible that by adding more enzyme (or bacterial cells) to the diluted sewage in the ordinary 5-day test the effective oxygen absorption may be brought about in a much shorter time, say one or two days. In working out such a method, however, it would be necessary to pay strict attention to the controls.

Conclusions.

1. The oxygen absorption of sewage sterilised by heat or by filtration is generally negligible.

2. A vigorous oxygen absorption takes place when sterile sewage is inoculated with a non-sterile crude sewage seeding, a suspension of washed sewage organisms (mixed) or a suspension of washed cells obtained from a single strain of certain bacteria.

3. The oxygen absorption due to non-sterile sewage can be inhibited by the addition of $M/1000$ KCN or of 5 % urethane.

4. The addition of a formaldehyde-treated suspension of *Ps. fluorescens*, which is sterile but enzymically active, will result in oxygen absorption by sterile sewage.

5. The oxygen absorption of sewage is due largely to the presence of bacterial enzymes, which may be associated with either living or dead cells, or possibly cellular débris.

The investigations recorded above were carried out as part of a programme of research instituted by the Water Pollution Board of the Department of Scientific and Industrial Research. Our thanks are due to the Engineers of Croydon and Finchley for supplying us regularly with the sewage used in this investigation.

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XXIX. THE "STABILITY TEST" OF SEWAGE AND ITS RELATION TO ENZYME ACTIVITY.

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The Rideal-Stewart 5-day biochemical oxygen demand test when applied to the study of changes taking place in any sewage purification system—experimental or otherwise—suffers from several drawbacks, not the least of which is the length of time required. This is a serious handicap to research work, and upon the suggestion of Mr Standfast, who is working on problems of sewage purification by "activated sludge" for The Water Pollution Research Board, the writer undertook an enquiry into the so-called "stability test" [Standard Methods of Water Analysis, 1925]. In this test 150 cc. of the sewage to be examined are incubated at 22° in a 150 cc. bottle, closed with a water seal, together with 0·4 cc. of a 1/2000 methylene blue (zinc salt) solution and its "stability" assessed from the time required, in days, for the complete decoloration of the dye. In this form it appears that the test presents certain possibilities of error; firstly the time taken for the methylene blue reduction to take place will depend to some extent on the amount of oxygen already dissolved in the sewage and secondly the concentration of methylene blue is so low that any error produced as a result of this variability in the amount of oxygen dissolved will be very significant, especially as the reducing system is generally such a weak one, taking several days for completion. These sources of error can be avoided by using the Thunberg technique, adding a larger concentration of methylene blue and shortening the time required for reduction by incubating at a higher temperature.

The reducing power of sewages as measured by methylene blue.

In these experiments ordinary Thunberg vacuum tubes were employed. The tubes were evacuated at the water-pump and incubated at 24°, 37° or 45°, the time required for the reduction of the dye being noted. Using a strong sewage containing a fair amount of suspended matter the time required for reduction is comparatively short and diminishes with rise of temperature; when however the crude sewage is filtered through ordinary filter-paper and the filtrate is used the reduction time is found to be very much prolonged. If the crude sewage is boiled or filtered through a Seitz bacteriological filter no reduction takes place (Table I).

From these results it appears that the main reducing activity of the crude sewage is associated in some way with the suspended matter of the sewage, and that it is destroyed by boiling. The addition of formate to the unboiled sewage systems brings about a shortening of the reduction time (Table I). The three experiments cited where the methylene blue was in the lower concentration (1/630,000), that usually employed in sewage analysis, give similar results although even here little or no reduction of the dye was noted with the Seitz-filtered sewage. The reducing power of the suspended matter in sewage was next

Table I.

Each Thunberg tube contains 4 cc. sewage preparation, 1 cc. of $M/20$ formate (p_H 7·2) or distilled water, 1 cc. p_H 7·4 buffer and 1 cc. of methylene blue solution (1/5000 or 1/90,000 as stated), the latter concentration being that usually employed in the sewage "stability test." The tubes were incubated at various temperatures and the time, R.T., taken for the dye to become reduced noted.

Temp. of incubation	Sewage preparation	R.T.
24°	Crude sewage + Water + M.B. (1/35,000)	60 minutes
	Filtered C.S. + Water + M.B. (1/35,000)	Not reduced after 5 days
	Crude sewage + Formate + M.B. (1/35,000)	40 minutes
	Filtered C.S. + Formate + M.B. (1/35,000)	1–2 days
37°	Crude sewage + Water + M.B. (1/35,000)	51 minutes
	Filtered C.S. + Water + M.B. (1/35,000)	Not reduced after 5 days
	Crude sewage + Formate + M.B. (1/35,000)	36 minutes
	Filtered C.S. + Formate + M.B. (1/35,000)	8 hours
45°	Crude sewage + Water + M.B. (1/35,000)	22 minutes
	Filtered C.S. + Water + M.B. (1/35,000)	Not reduced after 5 days
	Seitz-filtered C.S. + Water + M.B. (1/35,000)	" " "
	Boiled C.S. + Water + M.B. (1/35,000)	" " "
	Crude sewage + Formate + M.B. (1/35,000)	11 minutes
	Filtered C.S. + Formate + M.B. (1/35,000)	3 hours
	Seitz-filtered C.S. + Formate + M.B. (1/35,000)	Not reduced after 5 days
	Crude sewage + Water + M.B. (1/630,000)	5 minutes
	Filtered C.S. + Water + M.B. (1/630,000)	40 hours
	Seitz-filtered C.S. + Water + M.B. (1/630,000)	Not reduced after 5 days

examined. A litre of crude sewage was filtered and the deposit on the filter-paper washed with distilled water and made into a suspension with 100 cc. of water. The times required for 1 cc. of this suspension to reduce 1 cc. of 1/5000 methylene blue solution in the presence of crude or sterilised sewage or water only are given in Table II.

Table II.

Sludge, *i.e.* the suspended matter, of 1 litre of crude sewage, washed and suspended in 100 cc. distilled water. Each tube contained 7 cc., including 1 cc. p_H 7·4 buffer, 1 cc. 1/5000 M.B., 4 cc. of the substrate (C.S., *etc.*) noted below and 1 cc. of sludge preparation. The tubes were incubated at 45°. As the sewages used were from the same samples as were used in the experiments cited in Table I, the control reduction times without sludge may be taken from this table.

	Substrate	R.T.
Sludge	Distilled water	180 minutes
"	Crude sewage	18 "
"	Boiled C.S.	52 "
"	Seitz-filtered C.S.	34 "
Boiled sludge	Water	∞
" "	Boiled C.S.	∞

Thus it is clear that the suspended matter of sewage has a reducing power of its own which, however, is greatly increased by the addition of crude or sterilised sewage. As is to be expected, the reduction time of the sludge in the presence of crude sewage is somewhat shorter than that in the presence of sterilised sewage, owing to the presence in the former instance of a greater quantity of active suspended matter. Boiled sludge, alone or in the presence of boiled sewage, exerts no reducing power. These results suggest that the soluble constituents of the crude sewage have no reducing power of their own, although if added to the suspended matter the reducing activity of the latter is enhanced. Since the activity is destroyed by boiling the suspended matter, it seems that we are

dealing with an enzyme system which, although capable of reducing methylene blue itself, nevertheless activates certain constituents found in solution in crude sewage as hydrogen donators.

As boiled crude sewage and sewage sterilised by passage through a Seitz filter exert little or no reducing effects themselves, although they will still act as sources of hydrogen donators to the suspended matter, it appears that the reduction of the methylene blue is entirely the result of this enzymic system. As there are many observations in the literature pointing to sulphydryl systems as the cause of the reducing activity of sewage towards methylene blue, a large number of crude sewages (all domestic) have been examined by the nitroprusside test, but a consistently negative result has been obtained. The tests have also been made at the point of entry of the sewage into the sewage farms on two occasions, but here again no sulphydryl reaction could be detected. When, however, a series of samples taken at different stages of purification were collected and subsequently stored at 0° in full stoppered bottles, *i.e.* anaerobically, for over a week, it was found that the sewages collected from the point of entry, from the detritus tank and from the sedimentation tank now exhibited a strong nitroprusside reaction, whilst the sewage obtained after passage through the filters and the final effluent still gave negative results. On shaking with air, the crude sewage no longer exhibited a positive reaction, whilst the time it took to reduce methylene blue under the conditions already described was considerably lengthened, although the reducing capacity was not completely lost (Table III).

Table III.

The reducing power of various sewages collected from a sewage farm and its relation to the presence of sulphydryl compounds. R.T. = the time required by 4 cc. of the sewage to reduce 1 cc. of 1/5000 methylene blue at 45° anaerobically. SH compounds were tested for by the nitroprusside reaction. C.S. = crude sewage, S.S. = sewage from sedimentation tank, F.S. = effluent from filter beds and F.E. = final effluent. The sign ∞ = not reduced in 5 days.

Examination	C.S.	S.S.	F.S.	F.E.
SH at time of collection	0	0	0	0
RT " "	120 min.	48 hr.	∞	∞
SH after storage "anaerobically" at 0° for 7 days	+	+	0	0
RT after 7 days at 0°	3 min.	4 min.	∞	∞
SH after subsequent shaking with air	0	0	—	—
RT after subsequent shaking with air	131 min.	>48 hr.	—	—

Sewage sludge as a source of dehydrogenases.

When "activated sludge" is collected from an activated sludge plant, washed thoroughly with water and resuspended in water, it may be used as a source of enzyme for examination by the Thunberg technique, as equal amounts can be readily withdrawn in a pipette provided the sludge suspension is thoroughly mixed immediately before withdrawal. If such a washed sludge is examined in the presence of a large number of substrates, it is found that a considerable number of the latter become oxidised, *i.e.* the methylene blue is reduced. The results given in Table IV show that in addition to crude sewage, boiled crude sewage and crude sewage filtered by passage through a Seitz filter, many other substances such as formate, lactate, succinate, glucose, fructose, glycerol and glutamate will act as hydrogen donators, whilst yet other substances such as nitrate, chlorate, fumarate, malate, possibly aspartate and a final effluent from a sewage farm appear to act as hydrogen acceptors, for in their presence the

sludge takes much longer to reduce the methylene blue. Urea, phenol and sucrose, at least in the concentrations examined, appear to be unaffected (Table IV).

Table IV.

In this Table the enzymic activity of washed "activated sludge" as a source of dehydrogenases is recorded. The times for reduction of 1 cc. 1/5000 methylene blue when incubated anaerobically at 45° are given. 1 cc. of sludge suspension in distilled water was used as the enzyme preparation. 1 cc. of phosphate buffer, p_H 7·4, in each tube. All substrates previously adjusted to p_H 7·2, *e.g.* by neutralising acids with NaOH.

	Substrate	R.T.
Sludge	Water (distilled)	44 min.
,,	*M*/140 formate	19 ,,
,,	,, lactate	20 ,,
,,	,, succinate	27 ,,
,,	,, glucose	21 ,,
,,	,, fructose	20 ,,
,,	,, sucrose	50 ,,
,,	,, phenol	49 ,,
,,	*M*/3·5 glycerol	30 ,,
,,	*M*/7 glutamate	24 ,,
,,	,, urea	48 ,,
,,	Crude sewage (4 cc.)	17 ,,
,,	Boiled crude sewage	24 ,,
,,	Seitz-filtered C.S.	25 ,,
Boiled sludge	Water	No reduction after 5 days
,, ,,	Boiled sewage	,, ,, ,,
Sludge	*M*/3·5 fumarate	Not reduced 24 hr.
,,	,, malate	111 ,,
,,	,, aspartate	80 ,,
,,	*M*/10 nitrate	Not reduced in 5 days
,,	,, chlorate	,, ,,
,,	Final effluent (4 cc.)	,, ,,

In order to demonstrate more clearly the fact that such substances as nitrate, *etc.*, will act as hydrogen acceptors in the presence of "activated sludge," vacuum U-tubes were used. This apparatus consists of a vessel shaped like an upturned U with the two closed limbs about $\frac{3}{4}$-inch diameter but with an extension from the middle of the U, this extension being open to the air but capable of allowing evacuation and sealing, as it is finished off like the usual Thunberg tubes with a side-arm and a glass stopper. Each limb has a dilatation about two-thirds of the way up in order to stop liquid in one limb bubbling over during evacuation into the other limb.

In the actual experiment one limb contains a sludge reducing system, *e.g.* 1 cc. washed "activated sludge," 2 cc. p_H 7·4 buffer, 1 cc. *M*/20 formate and 1 cc. 1/5000 methylene blue solution, whilst in the other limb are placed 3 cc. of the substance to be tested as a hydrogen acceptor. The tube is evacuated, filled with nitrogen, evacuated again and then partially filled with nitrogen. It is then incubated at 45°. When the dye is reduced in the one limb its contents are partially tipped over into the other, some however remaining to act as a control, in case a small amount of oxygen still remains in the tube bringing about a temporary slight blueing. If the substance being examined acts as a hydrogen acceptor in the presence of the sludge the leucomethylene blue becomes re-coloured. That this activity is dependent upon the sludge can be shown by boiling the contents of the first limb after reduction has taken place; then on tipping no blue colour develops (Table V).

Table V.

Experimental details given in text.

Possible H acceptor	On tipping, without boiling	On tipping, after boiling
Crude sewage	No blue colour develops	—
Sewage, sedimentation tank	,, ,,	—
Sewage after filter beds	Blue colour develops	No blue colour develops
Final effluent	,, ,,	,, ,,
3 cc. *M*/2 fumarate	,, ,,	,, ,,
,, aspartate	,, ,,	,, ,,
,, malate	,, ,,	,, ,,
3 cc. *M*/100 $NaNO_3$	,, ,,	,, ,,
3 cc. *M*/1000 $NaNO_3$	,, ,,	,, ,,
3 cc. *M*/100 $KClO_3$	,, ,,	,, ,,

It is clear then from the results given in Table V that fumarate, malate, aspartate, nitrate and chlorate will act as hydrogen acceptors in the presence of "activated sludge". Furthermore, although crude sewage will not so act yet purified sewage obtained after passing through the filter-beds and the final effluent after land treatment appear to supply hydrogen acceptors to the system. Possibly the only hydrogen acceptor supplied by the effluents is nitrate, which was present in both the effluents examined, but we cannot assume, without proof, that this is the sole hydrogen acceptor present in all sewage effluents which give low oxygen demand figures.

Sludges obtained both from a sewage sedimentation tank and from a sewage humus tank were examined by the Thunberg technique to see whether they differed from "activated" sludge. In Table VI a series of results is given comparing the activity of the three sludges, but it should be realised that no strict comparison is possible owing to the difficulty of securing equal suspensions. It is seen that the three sludges exhibit the same type of dehydrogenase activity.

Table VI.

Details as in previous tables using the Thunberg technique. Each tube contains 1 cc. 1/5000 methylene blue solution, 1 cc. p_H 7·4 buffer, 1 cc. sludge and, except the water control, 1 cc. *M*/20 formate, the volume being made up to 7 cc. with a sewage preparation or water.

Substrate	Activated sludge	Sedimentation tank sludge	Humus tank sludge
Water	34 min.	6 hr.	180 min.
M/140 formate	20 ,,	53 ,,	38 ,,
,, +Crude sewage	13 ,,	19 ,,	23 ,,
,, +Sedimentation tank sewage	15 ,,	20 ,,	24 ,,
,, +Effluent from filters	133 ,,	>6 hr.	>6 hr.
,, +Final effluent	>3 hr.	>6 ,,	>6 ,,
,, +Boiled sludge (control)	>3 ,,	6 ,,	6 ,,

Certain other enzymes in "activated sludge."

"Activated sludge" taken from the School experimental tank has been examined for the presence of certain other enzymes concerned in oxidation processes. When tested for indophenol oxidase by the method of Keilin [1929] a positive reaction was obtained, which was inhibited by the presence of KCN. In the actual test 2 cc. of sludge suspension were shaken for 10 min. in an open test-tube with 1 cc. buffer p_H 7·4, 1 cc. of water or KCN and 0·5 cc. of freshly

prepared 1 % *p*-phenylenediamine solution. Boiled sludge gave no reaction. Crude sewage and filtered crude sewage gave a doubtful reaction whilst sewage passed through a Seitz filter gave a negative result (Table VII).

Table VII.

Examination of "activated" sludge and crude sewage for the presence of oxidase, peroxidase, catalase and urease. Experimental details in the text. A – sign signifies that no test was made.

	Sludge	Boiled sludge	Crude sewage	Filtered C.S.	Seitz-filtered C.S.
Indophenol oxidase	+	0	?	?	0
Peroxidase	?	0	–	–	–
Catalase	0	0	0	–	–
Urease	+	0	–	–	–

Sludge was examined for the presence of peroxidase, using both 1 % benzidine and guaiacum as colour reagents. A very doubtful reaction was obtained. Catalase appeared to be absent from both crude sewage and sludge. Sludge was tested for the presence of urease by mixing equal volumes of washed sludge suspension and urea solution, adjusting to p_H 7·0, using phenol red as indicator and incubating at 37°. After half an hour the solution had become distinctly alkaline owing to the decomposition of the urea. A control tube, using boiled sludge, showed no change in reaction. Urease therefore appears to be present in the sludge.

A modified methylene blue test for the examination of sewage and sewage effluents.

It has already been shown in this paper that a strong crude sewage acts as a hydrogen donator in the presence of sewage sludge and can thereby effect a rapid reduction of methylene blue anaerobically, whilst in the absence of the sludge, especially if it be freed from suspended matter, its reducing effect is comparatively slight. On the other hand all good sewage effluents that have so far been examined act as hydrogen acceptors in the presence of sludge and therefore take longer to reduce the dye, if it is reduced at all, than does a sludge control. It therefore appears that by examining the reducing power of a sewage in the presence of sludge we can form some idea as to whether it is extremely foul or has been oxidised to more stable products. As one is most anxious in a sewage examination to learn whether an effluent is a good one or not the reducing activity of the system can be further strengthened by the addition of another donator such as formate. In this way the oxidising action of the effluent in the presence of the sludge must be sufficiently great to balance both the reducing effect of the sludge itself and of the formate system to secure that the dye does not become reduced. In Table VIII are given the results of a series of examinations of sewage obtained from three different farms, using the Thunberg technique and incubating at 45°. Experiments were carried out with the sewages alone, with sewage and sludge mixtures, with formate sewage mixtures and with formate-sewage-sludge systems.

By this modified methylene blue method it appears possible to differentiate readily between a crude sewage and a final effluent of low oxygen demand value and containing nitrate. The most satisfactory results are obviously those obtained using sludge. Whether it is possible to distinguish an effluent with a 5-day oxygen demand figure of less than two parts per 100,000 from one with a value

Table VIII.

The time taken for 4 cc. of various sewages obtained from 3 sewage farms of different types to reduce 1 cc. of methylene blue when incubated at 45° anaerobically either alone or with washed "activated" sludge and/or 1 cc. *M*/140 formate. The total volume was made up to 7 cc. with water. The 5-day Biochemical Oxygen Demand values for these sewages, determined by the Rideal-Stewart method, are given for comparison. Similarly, the amount of nitrate present in the sewages has been assessed by the phenolsulphonic acid method and is given as parts of nitrate-nitrogen per 100,000 parts of sewage. The sign ∞ = not reduced in 5 days. When times of reduction are given in days, they are approximate. The sewages have been filtered through ordinary filter-paper.

Sewage	R.T. sewage alone	R.T. sewage + formate	R.T. sewage + sludge	R.T. sewage + sludge + formate	5-day B.O.D.	Nitrate
Ordinary biological treatment						
Control (with sewage)	—	—	180 min.	33 min.	—	—
Crude sewage	1 day	1 day	70 „	28 „	16·6	0
Sedimentation tank effluent	2 days	2 days	73 „	31 „	16·3	0
Percolating filters effluent	∞	∞	2 days	18 hr.	1·7	1·52
Final effluent	∞	∞	2 „	16 „	1·5	1·32
Activated sludge plant.						
Control (without sewage)	—	∞	73 min.	42 min.	—	—
Crude sewage	∞	2 hr.	35 „	24 „	21·3	0
Diffusion tank effluent	∞	4 days	38 „	29 „	12·8	0
Simplex tank effluent	∞	4 „	40 „	29 „	13·6	0
Channel tank effluent	∞	4 „	42 „	28 „	14·0	0
Final effluent after filters	∞	∞	∞	∞	1·63	2·78
Chemical treatment.						
Controls (without sewage)	—	—	73 „	42 „	—	—
Crude sewage	3 days	2 days	70 „	33 „	19·5	0
$Fe_2(SO_4)_3$ tank effluent	∞	∞	128 „	40 „	10·8	0
Final effluent after filters	∞	∞	∞	∞	0·5	2·25

a little above this amount cannot be decided from the above experiments. The method appears to be of some use in following the steps of progressive sewage purification, as can be seen from Table IX. In these experiments quantities of a sewage-sludge mixture were withdrawn at various times from the experimental activated sludge tank, filtered and examined by the methylene blue technique. The results obtained were compared with the 5-day oxygen demand and nitrate-nitrogen figures.

Table IX.

Reduction times, 5-day oxygen demand figures and nitrate-nitrogen contents of sewage at different stages of purification by activated sludge in an experimental tank. Details as in Table VIII.

Period of treatment (*i.e.* aeration)	R.T. sewage	R.T. sewage + formate	R.T. sewage + sludge	R.T. sewage + sludge + formate	B.O.D.	Nitrate
(Crude sewage)	4 days	3 days	15 min.	12 min.	17·8	0
5 min.	∞	4 „	21 „	14 „	17·2	0
30 „	∞	∞	28 „	17 „	9·1	0
60 „	∞	∞	73 „	22 „	6·5	0
90 „	∞	∞	140 „	23 „	5·4	0
2 hr.	∞	∞	> 7 hr. / < 22 „	172 „	4·8	0·18
3 „	∞	∞	„	186 „	4·4	0·3
4 „	∞	∞	„	> 6 hr. / < 20 „	3·6	0·46
5 „	∞	∞	„	„	2·5	0·56
6 „	∞	∞	28 hr.	„	2·1	0·9
Control (without sewage)	—	—	74 min.	33 min.	—	—

It is clear from the results cited in Table IX that it is not possible to decide by the methylene blue test when an effluent will have a B.O.D. of less than two parts per 10^5, but the test with formate and sludge in addition to the sewage seems to offer a fairly reliable guide as to whether a sewage approximates in strength to that of a good effluent. There is every reason to think that this test will be useful in supplying a fairly rapid method for assessing the strength of a sewage, although not so satisfactory as the ordinary B.O.D. method if the actual strength is required. As a rough guide and from a large number of examinations similar to those detailed in Table IX one might take a reduction time twenty times as long as that of the sludge-formate control as the time corresponding approximately to that obtained with a satisfactory effluent. That this approximation would hold with a good effluent which did not contain nitrate seems to be unlikely but no experiments have, as yet, been made with such an effluent. The amount of nitrate present will obviously affect the result of the test, but other factors, such as the concentration of hydrogen donators and possibly other hydrogen acceptors, are also important. A less accurate test may be carried out in bottles without evacuation, using larger quantities of sludge and sewage. Specific gravity bottles of 50 cc. capacity form useful vessels. In the actual experiment 40 cc. of sewage were incubated at 37° with 5 cc. of sludge and 5 cc. of 1/5000 methylene blue. A series of results is given in Table X. The presence of sludge tends to minimise the opposing effect of any dissolved oxygen but it is found that results are subject to greater variation by this method, partly owing to the fact that the rate of diffusion of the dye becomes important as the sludge quickly settles to the bottom.

Table X.

In this Table the reduction times of sewage-sludge systems are compared by the vacuum tube and the bottle methods. The details of the former method are the same as previously described, the tube containing 4 cc. of the sewage, 1 cc. of washed sludge, 1 cc. of 1/5000 methylene blue solution and 1 cc. of water, and being incubated at 45°. In the bottle method 50 cc. specific gravity bottles were used and these were incubated at 37°. They contained 40 cc. of sewage, 5 cc. of sludge and 5 cc. of 1/5000 methylene blue solution. The B.O.D. and nitrate values were determined by the usual methods. The sewages were from the experimental activated sludge tank, and are described in the Table simply by the times given at the head of the columns. These times indicate the extent of the aeration treatment to which the sewage-sludge mixture has been submitted.

	Control	C.S.	1 hr.	2 hr.	3 hr.	5 hr.
R.T. vacuum tube, 45°	11 min.	6 min.	20 min.	10 hr.	33 hr.	35 hr.
R.T. bottle, 37°	5 hr.	2 hr.	10 hr.	14 ,,	18 ,,	20 ,,
B.O.D.	—	18·0	6·3	4·6	3·9	2·6
Nitrate	—	0	0·04	0·50	1·24	2·00

It may be concluded that a modification of the methylene blue "stability test," whereby a sewage-sludge-formate-methylene blue system is incubated anaerobically at 45°, can be used with advantage whenever the "stability test" is of value. It may also prove of use for following sewage research, although its general application will always remain limited. Its advantage over the "stability test" lies mainly in its comparative rapidity and its avoidance of error due to dissolved oxygen (as this is largely lost during evacuation).

Summary.

(1) The reducing power of crude sewage towards methylene blue is shown to depend largely upon the enzymic activity of suspended matter in the sewage. The sludge, besides possessing a marked reducing power of its own, also activates

substances present in solution as hydrogen donators. Both reducing effects are destroyed by heating the sludge to 100° for 15 minutes.

(2) Sulphydryl compounds are rarely, if ever, found in sewage unless this has been kept anaerobically for some time.

(3) Strong sewages contain potential hydrogen donators, and good effluents contain potential hydrogen acceptors, of which nitrate is usually one.

(4) Besides its dehydrogenase activity some other enzymic properties of the sludge are examined.

(5) A modified "stability" test is described. The period of the test is shortened by incubation at 45° and by the addition of washed sludge and a hydrogen donator such as formate. The sewage is incubated in vacuum tubes together with 1 cc. of 1/5000 methylene blue solution, formate and washed sewage sludge, and the time required for complete reduction of the dye is compared with that of a control tube not containing the sewage. Variations of this test are given.

My thanks are due to Mr Standfast for allowing me to use material from his experimental activated sludge tank, and to the Engineers of Croydon and Finchley for supplying me with the sewage used in this investigation.

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XXX. IMPROVED METHODS FOR THE ISOLATION OF METHIONINE AND ERGOTHIONEINE.

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(*Received December 30th, 1932.*)

Methionine.

In a recent paper [Pirie 1932, 1] a method for the preparation of methionine was described. The principal difference between this method and that of Mueller lay in the use of mercuric acetate instead of mercuric sulphate, to avoid the inhibiting effect of the sulphate ion in an amino-acid mixture on the precipitation of methionine. The chloride ion has a similar interfering action and for the preparation of methionine it is best therefore to use an enzymic hydrolysate of caseinogen although a sulphuric acid hydrolysate from which the acid has been removed with baryta is fairly satisfactory.

Of the mercuric salts that are available commercially the acetate is the most suitable for the isolation of methionine; it seemed possible however that some other anion might improve the precipitation. After some search it was found that phosphotungstic acid, although it does not itself precipitate methionine either from pure solution or from an amino-acid mixture, increases the efficiency of precipitation by mercuric acetate. The mercury-phosphotungstic precipitate has the further merit of being more easily decomposed by baryta than the precipitate obtained with mercury alone; there is therefore no risk of racemisation during the extraction.

The amino-acid mixture recovered from this mercury-phosphotungstic precipitate is very similar to the mixture obtained by the original mercuric acetate precipitation. In each case the N/S ratio is about 3 and there is present no amino-acid, other than methionine, which is precipitated by mercuric chloride in slightly acid solution. The mixture has only been investigated in a superficial manner, but it seems to contain considerable quantities of phenylalanine- and proline-containing peptides. From this mixture the methionine may be precipitated directly by mercuric chloride, but if this is done, very much more mercuric chloride must be added than is necessary if the mixture is first fractionated with alcohol. By the latter means also a rather purer product is obtained.

Caseinogen and egg-albumin are the proteins which are most readily available for the preparation of methionine. The latter gives a very much larger yield (2·5 %) but it is more expensive. Furthermore, it is necessary to coagulate the commercial dried egg-white (by dissolving it in water and heating), partly to get rid of non-protein material and partly to facilitate the enzymic hydrolysis. For these reasons caseinogen is probably the most convenient starting-point.

Experimental.

1 kg. of caseinogen is suspended in 10 litres of water and the p_H adjusted to 8·4 with sodium hydroxide; a suitable amount of pancreatic extract is added and the mixture incubated at 37° in the presence of a little toluene, more sodium hydroxide being added from time to time to keep the digest at p_H 8·4. After 3 weeks it is brought to p_H 6 with acetic acid and allowed to stand in a cool place for a day; the tyrosine and undissolved materials are removed by filtration through a calico bag which is finally squeezed out in a press.

700 cc. of a 30 % solution of mercuric acetate in 2 % acetic acid are added to the clear filtrate; this will cause a slight precipitate to form most of which redissolves on vigorous stirring. 1000 cc. of 30 % phosphotungstic acid solution are now added in a thin stream with vigorous stirring, followed by 400 cc. more of the mercuric acetate solution. If the addition of a small amount of phosphotungstic acid to a filtered test portion causes further precipitation a further 100–200 cc. should be added to the main bulk of the preparation. If large amounts of either mercuric acetate or phosphotungstic acid are added a further precipitate will always be obtained but this is of no value in the preparation of methionine.

The precipitate is sucked as dry as possible on Büchner funnels and washed by suspending in 2–3 litres of water and filtering again. It is resuspended in water and heated to boiling while solid barium hydroxide is added, the addition being continued until the p_H remains at about 8·5 for 5 minutes without further addition of barium hydroxide.

The barium phosphotungstate is filtered off and extracted again with a litre of water and enough barium hydroxide to bring the p_H of the boiling suspension to about 9. The combined filtrates are brought to p_H 5 with sulphuric acid and filtered; a little kieselguhr on the filter-paper will ensure clear filtration. The filtrate will generally not distil easily *in vacuo* on account of frothing; it is therefore concentrated in a large evaporating basin over a flame until the volume is about 300 cc.; at this stage it is filtered from a trace of a mercury compound. The filtrate is evaporated on a boiling water-bath until a considerable amount of white solid separates (about 200 cc.). To this hot solution 400 cc. of alcohol are added and the mixture is left overnight at 0°. The solid is filtered off; if dried it should weigh about 50 g. and contain 6 % of sulphur. The filtrate will deposit more solid if it is distilled *in vacuo* and treated with more alcohol; care must be taken however that the concentration of alcohol does not exceed 80 % or a gum will be thrown out which has a very low sulphur content and interferes with the precipitation in the next stage. A little sodium hydroxide may with advantage be added to the alcoholic solution to bring its p_H to about 6. The precipitate obtained is mixed with the other lots. The alcoholic filtrate still contains some sulphur (estimated by the method recently described [Pirie, 1932, 2]); this is not easily recoverable because the N/S ratio may be as high as 15.

The solid thrown out by alcohol is dissolved in 300 cc. of hot water and the solution, filtered if necessary, is added to a boiling solution of 200 g. of mercuric chloride in 300 cc. of water. When cool a putty-like mass separates. The mixture is cooled in a freezing mixture and the solid separated as well as possible from the mother-liquor; the former is boiled up with 200 cc. of water and again chilled. Methionine is isolated from this mercury precipitate in the usual way [Pirie, 1932, 1].

The yield by this method is very constant; from 1 kg. of caseinogen 9–10 g. of pure methionine are obtained directly and a further 2–3 g. may be obtained by working up the mother-liquors in the manner outlined in the previous paper.

Ergothioneine.

It has long been known that thiolglyoxalines, like other mercaptans and like thiourea and its derivatives, form cuprous compounds which are insoluble in dilute acids [Wohl and Marckwald, 1889; 1892]. Although the cuprous compound of ergothioneine might have been expected to be a derivative well suited for the isolation and characterisation of that substance it has only very recently been prepared. Williamson and Meldrum [1932] have used the cuprous compound for the isolation of ergothioneine from blood; their method is simpler than any of the older ones and it gives a better yield. Blood, however, is a less convenient source of ergothioneine than ergot and the present paper describes a method, based on that of Williamson and Meldrum, for the isolation of the base from this material.

I have already described [Pirie, 1931] the cuprous derivatives of a number of mercaptans and have put forward the theory that only substances which contain a peptide linkage are soluble in the presence of an excess of cuprous oxide. Ergothioneine is another case in point for it is not soluble in dilute acid suspensions of an excess of cuprous oxide; the cuprous compound is however soluble in water. Cuprous ergothioneine therefore, like cuprous acetylcysteine, goes into solution if it is washed with distilled water; this fact is used to separate ergothioneine from a purine-like substance, similar to that already mentioned as a constituent of yeast [Pirie 1931, p. 626], which is precipitated by cuprous oxide from an aqueous extract of ergot.

The cuprous compound of ergothioneine is much less stable than that of glutathione and other similar mercaptans. It can be kept for some time in a vacuum but in air it rapidly goes grey and finally black. For this reason accurate analyses are impossible but the cuprous compound, on decomposition with hydrogen sulphide, gives rise to about half a molecule of sulphuric acid for each molecule of ergothioneine. This is in agreement with the structure that would have been expected *a priori* $(C_9H_{15}O_2N_3S)_2.SO_4Cu_2$.

Experimental.

900 g. of ground ergot are added to 4 litres of water and boiled for 5–10 minutes. The mixture is filtered through calico and the residue pressed. The solid is then re-extracted with a further 4 litres of water in the same way. Basic lead acetate solution is added, in considerable excess, to the combined milky extracts and the precipitate removed by filtration. This filtration is rather slow; it is advisable therefore to allow the precipitate to settle and to pour off as much as possible of the supernatant liquid. About 9 litres of clear filtrate will be obtained at this stage, and 200 cc. of concentrated sulphuric acid are added with thorough stirring. Lead sulphate is removed after about half an hour by filtering through kieselguhr. This filtrate is perfectly stable but the processes described in the next paragraph should be carried through without any avoidable delay.

Cuprous oxide suspension is added gradually until the initially formed white precipitate has become almost black; after about half an hour the supernatant fluid is discarded and the thick suspension centrifuged. The solid is stirred up thoroughly with 900 cc. of 0·3 *N* sulphuric acid and centrifuged again. It is now stirred up with distilled water and centrifuged; this extraction is repeated five times so that in all 4–5 litres of extract are obtained. Enough sulphuric acid is added to make it 0·5 *N*; this causes slight precipitation of cuprous ergothioneine, and the remainder of the ergothioneine is thrown out by the addition of cuprous oxide as before. This precipitate is allowed to settle somewhat and then filtered

off; it is suspended in 200 cc. of water to which a gram of barium chloride has been added and decomposed with H_2S.

The suspension of cuprous sulphide is heated nearly to boiling and filtered; the filtrate should contain a slight excess of sulphuric acid; if there is an excess of barium this must be removed with a little sulphuric acid. The filtrate is distilled *in vacuo* to small bulk and then allowed to crystallise in a vacuum desiccator after addition of a few drops of concentrated hydrochloric acid. The filtrate from the first crop is taken to dryness and the residue crystallised from a few cc. of hot water. The two lots of crystals are mixed and recrystallised from the minimum amount of boiling water. These crystals are dihydrated ergothioneine hydrochloride; when dried *in vacuo* 1·5–1·6 g. of the anhydrous hydrochloride are obtained. (Found: N 15·95; S 11·90; Cl 13·65 %. Calculated: N 15·82; S 12·05; Cl 13·37 %.) From the mother-liquor 0·2–0·4 g. of anhydrous ergothioneine may be recovered by evaporating to dryness, taking up in hot alcohol, filtering and adding a few cc. of pyridine. A crystalline precipitate separates on cooling; this is washed with alcohol and consists of pure anhydrous ergothioneine.

The total yield is therefore 0·18 % of the anhydrous base; the yields from ergot already given in the literature are: Tanret [1909] 0·1 % of the hydrochloride and Eagles [1928] 0·065 % of the free base. In each case the method of isolation is very much more laborious than that described in this paper.

Summary.

Methionine is isolated from an enzymic hydrolysate of caseinogen by precipitation with mercuric acetate and phosphotungstic acid (in one operation), followed by fractionation with alcohol and precipitation with mercuric chloride. The yield is 1·2 %. Ergothioneine is prepared by means of its cuprous derivative from an aqueous extract of ergot. The yield is 0·18 %.

I am very grateful to Sir F. G. Hopkins for the interest he has shown in this work.

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XXXI. A SURVEY OF ANTHOCYANINS.

III. NOTES ON THE DISTRIBUTION OF LEUCO-ANTHOCYANINS.

By GERTRUDE MAUD ROBINSON and ROBERT ROBINSON.

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(*Received December 31st, 1932.*)

In the course of an investigation of the pigment of young vine leaves, Rosenheim [1920] noted the presence in the leaves of a water-soluble colourless substance which yielded a colouring matter regarded as anthocyanidin on treatment with hot 20 % hydrochloric acid.

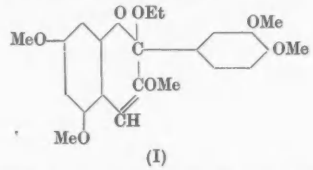
This important discovery threw light on the cause of some unexplained observations of Willstätter and Nolan [1915] who found that a methyl alcoholic hydrogen chloride extract of *Rosa gallica* showed a gradual increase of tinctorial intensity to about twice its original value on keeping; this change occurred in the absence of oxygen.

Rosenheim called the new class of colourless generators of anthocyanidins "leuco-anthocyanins" and advanced the hypothesis that they are glucosides of the pseudo-bases of the anthocyanidins. He found that leuco-anthocyanin occurs in the skins of white grapes and that the formation of anthocyanidin on hydrolysis is just as rapid in a stream of carbon dioxide as in air.

The present authors, having meanwhile found that colourless progenitors of anthocyanidin occur in almost every kind of plant material, enquired of Dr Rosenheim as to whether a further publication was contemplated. The reply was, respecting the immediate future, in the negative; but Dr Rosenheim kindly communicated that "he has followed up the relative distribution of leuco-anthocyanins, anthocyanins and flavones in a vine during a whole year from spring to autumn. He examined the young and mature (pigmented) leaves, the ripe and unripe fruit, the seeds, bark, wood and roots; he has also isolated a white powder containing leuco-anthocyanin from the skins of muscat grapes and noted the occurrence of leuco-anthocyanin in various parts of rose trees."

Although we propose to retain the name leuco-anthocyanin for the colourless substances which we have found in a variety of woods, seeds and other parts of plants, it is unfortunate in one respect in that the prefix usually connotes reduction of a dyestuff and in this case it is probable that the leuco-anthocyanin and the anthocyanidin are in the same state of oxidation. The leuco-anthocyanins are normally stable in the presence of 10–15 % aqueous hydrochloric acid in the cold, although alcoholic hydrogen chloride of similar concentration, or even weaker, brings about gradual formation of anthocyanidin. This degree of stability to the action of a mineral acid excludes, in our opinion, the hypothesis of Rosenheim that the leuco-anthocyanins are saccharides of the anthocyanidin (or substituted anthocyanidin) pseudo-bases. Substitution of the hydrogen of the carbinol hydroxyl by alkyl groups does not protect the pseudo-bases derived from flavylium salts against the action of even quite weak acids.

Thus dilution of an alcoholic solution of *O*-pentamethylcyanidin chloride by absolute alcohol causes slow decoloration and such a solution must contain the ethyl ether of the pseudo-base (I or II). Reconstitution of the anthocyanidin



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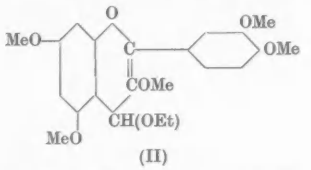
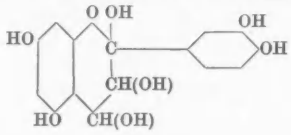


(II)

colour-salt is rapidly brought about by the addition of 5 % aqueous hydrochloric acid. Other anthocyanidins and also anthocyanins behave similarly, but the naturally occurring members of the group are not easily changed to pseudo-bases in pure alcohol; they tend to give rather stable colour-bases.

In order to avoid this difficulty we suggest that the leuco-anthocyanins contain the group —CH(OH).CH(OH)— at position (3 : 4) in the pyran ring. Thus the immediate precursor of cyanidin chloride is assumed to be (III), ring-chain tautomeric with (IV); conversion to the anthocyanidin then demands dehydration (loss of hydroxyl at 4 and hydrogen at 3) and as the hydroxyl at 4 is in the



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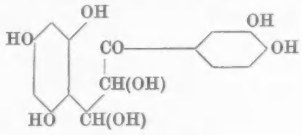


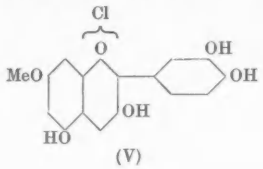
(IV)

β-position to a carbonyl group this process will certainly proceed in the direction leading to flavylium salt formation rather than in that leading to a flavone. In the actual leuco-anthocyanins any of the hydroxyl groups may bear carbohydrate residues or be acylated.

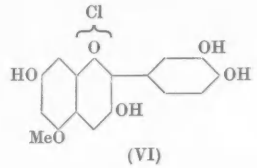
Although the greater number of the leuco-anthocyanins are readily water-soluble, a certain number of them are obtained in the first instance in colloidal solution and after flocculation cannot be redissolved. We suggest that in these cases the potential anthocyanidin molecule is attached to a polysaccharide (see *Eucalyptus marginata*).

The actual details will be found in the experimental section and the plan adopted has been the identification of the anthocyanidins obtained from the leuco-anthocyanins, the methods being those of Part I [1931], and Part II [1932] of this series.

In order to compare the properties of a new anthocyanidin from the wood of *Peltogyne* spp. and from the wood of *Copaifera pubiflora* with authentic specimens, new syntheses of rhamnetinidin chloride (V) and 5-*O*-methylcyanidin chloride (VI) have been effected.



(V)



(VI)

The condensation of 4-methoxy-2 : 6-dihydroxybenzaldehyde and ω-3 : 4-trihydroxyacetophenone with the help of hydrogen chloride gave a product (V) having exactly the same properties as the rhamnetinidin chloride which Robertson and Robinson [1927] prepared by the reduction of rhamnetin. Hence the statement of Malkin and Nierenstein [1930] that rhamnetinidin chloride is a *bis*flavylium salt cannot be accepted. We have long been of the opinion that the major (sparingly soluble or insoluble) products of the reduction of flavonols in acid solution are produced from pinacols but these substances are accompanied by true crystallisable anthocyanidins in smaller relative amount. Thus the preparation of cyanidin chloride from quercetin by reduction under the conditions prescribed by Willstätter and Mallison [1914] is reproducible and, as in the case of rhamnetinidin, we have identified cyanidin chloride made in this manner with cyanidin chloride synthesised by way of benzoylcyanidin chloride [Robertson and Robinson, 1928].

The remarkably wide distribution of leuco-anthocyanins opens up many interesting questions of biogenesis and possible physiological function, but it is too early to speculate even on the nature of the relation between the leuco-anthocyanins and the anthocyanin pigments of flowers. This is certainly not so simple and direct as it appears at first sight.

Experimental.

Extraction of leuco-anthocyanins. In many cases the leuco-anthocyanin can be extracted by means of distilled water, various woods yielding acid solutions. Boiling 1 % hydrochloric acid has, however, usually been employed because this also extracts, preserves and indicates free anthocyanin or anthocyanidin. Should free anthocyanin be present it is possible to effect the complete hydrolysis of the glucosidal pigment before attacking the leuco-anthocyanin. The anthocyanidin is then removed by means of amyl alcohol and the separated aqueous solution, on concentration by boiling, affords further anthocyanidin, the result of decomposition of the leuco-anthocyanin. Extraction by means of alcohol or alcoholic hydrogen chloride was always more effective than the use of water.

In many cases the anthocyanidins required drastic purification, and removal of impurities by extraction with ethyl acetate was frequently practised. When ethyl alcohol or ethyl acetate was employed especial care was taken to remove every trace of these solvents before carrying out distribution-ratio experiments with the anthocyanidin solutions.

Summary of results.

Aesculus hippocastanum. The crushed endocarp was extracted with boiling 1 % hydrochloric acid, the filtered solution made up to 10 % hydrochloric acid, boiled for 2 minutes and the anthocyanidin purified. This is the standard process unless otherwise stated and in this case the anthocyanidin proved to be cyanidin.

Arachis hypogaea. The testa is rich in leuco-anthocyanin affording cyanidin.

Baphia nitida. Barwood contains a leuco-anthocyanin which gives rise to an anthocyanidin not conforming to the usual tests and requiring further examination.

Bertholletea excelsa. The endocarp (shell of brazil nut) yields a little leuco-anthocyanin to boiling 1 % hydrochloric acid but treatment with hot 15 % alcoholic hydrogen chloride results in the formation of a relatively large amount of cyanidin chloride.

Caesalpinia granadillo. Results similar to those with *Baphia nitida* from the heartwood. Since this is a well-known redwood containing brazilin it is possible that the anomalous results are due to contamination with *iso*brazilein salts.

Castanea sativa. Endocarp. Leuco-A. → cyanidin.

Cocos nucifera. The fibres of the coconut contain much leuco-A. → cyanidin.

Copaifera pubiflora. The 1 % hydrochloric acid extract of the ground heartwood contains already some anthocyanidin of undetermined nature and this is removed by the minimum of amyl alcohol. In this case the leuco-anthocyanin can be almost completely extracted from the aqueous layer by means of ethyl acetate. It may therefore be non-glucosidic or at most monoglucosidic. The substance may be rendered to 1 % hydrochloric acid after addition of light petroleum to the ethyl acetate and, on boiling with 10 % hydrochloric acid, it affords an anthocyanidin tallying in every detail with peltogynidin (see *Peltogyne* below).

Diospyros sp. Ebony of W. African origin extracted by means of alcoholic hydrogen chloride gave anthocyanidin having the same reactions as that from *Caesalpinia granadillo*.

Entandrophragema macrophyllum. The leuco-A. of mahogany affords mainly delphinidin with indications of admixed methylated derivative or cyanidin.

Erica gracilis. The flowers, stem-wood and roots contain (apparently) the same leuco-A. yielding a complex (*i.e.* acylated) anthocyanidin which is Fe+. It is unusually difficult to hydrolyse by means of alkalis but, on prolonged treatment, cyanidin is produced.

Eucalyptus fascicula. The wood was extracted by means of cold 18 % alcoholic hydrogen chloride and the anthocyanidin thus produced was cyanidin. It was independently proved that the pigment arises from leuco-A.

E. marginata. Powdered jarrah gives an acid extract with cold distilled water, and on the addition of $\frac{1}{4}$ vol. of concentrated hydrochloric acid a colloid, which passes through an ordinary filter-paper, is precipitated. On shaking with ethyl acetate this is obtained in a more solid form, and after collection it cannot be redissolved in water. This solid material and also the residual aqueous solution afford delphinidin on treatment with hot 10 % hydrochloric acid.

The bark, sapwood and heartwood of this eucalypt contain leuco-A. → delphinidin; the cortex is the richest and the sapwood the poorest in potential anthocyanidin content.

E. tereticornis. The wood of this species also contains leuco-A. → delphinidin, but admixture with cyanidin or methylated delphinidin was indicated.

Fagus sylvatica. Beech-nuts contain a leuco-A. affording an unusual anthocyanidin which tallies with, but of course cannot be yet definitely identified with, 6-hydroxycyanidin chloride. This substance has recently been synthesised in collaboration with Dr E. H. Charlesworth (unpublished work). The Fe+ reaction is violet and the distributions and behaviour in the oxidation test are intermediate between cyanidin and delphinidin. The alkali colour reactions are much redder than with cyanidin or delphinidin (violet instead of blue; red-violet instead of blue-violet).

Helichrysum bracteatum. The red flowers contain a leuco-A. → complex anthocyanidin (Fe+).

Hydrangea hortensis. Faded flowers of a red variety were found to contain much leuco-A. → complex anthocyanidin. On treatment of the aqueous solution of the leuco-A. with sodium hydroxide, boiling half a minute, and then decomposing with hot 10 % hydrochloric acid, cyanidin chloride resulted. In this case it is clear that the leuco-anthocyanin yields an anthocyanidin different from that present in the flower-pigment since the latter is a delphinidin derivative (see Part II). Leuco-anthocyanin was also found in fresh green leaves.

Hymenaea Courbaril. The sawdust contains leuco-A. → cyanidin.

Juglans regia. The walnut is interesting in that the endocarp contains leuco-A. → 3-acylated cyanidin simulating delphinidin and the testa affords leuco-A. → acylated cyanidin → cyanidin.

Juniperus virginiana. The sawdust gave leuco-A. → cyanidin. In the case of *J. procera* the final anthocyanidin was cyanidin with some admixture, possibly of a methylated cyanidin.

Lathyrus odoratus. There seems to be a relation between the leuco-anthocyanin of the seeds of sweet-peas and the colour of the flowers, but an insufficient number of cases has been investigated to establish this.

"2 LO" (scarlet flowers with pelargonidin bioside) has a seed-coat containing leuco-A. → pelargonidin or peonidin and more probably the former.

"Model" (white flowers) gave leuco-A. → cyanidin.

"Olympia" (purple flowers) gave leuco-A. → petunidin or cyanidin—delphinidin mixture and probably the former. A remarkable feature is the high content of leuco-anthocyanin in all these seed-coats.

Mimusops Heckelii. The sawdust gave leuco-A. → cyanidin.

Payena utilis. Very little can be extracted from the sawdust by hot aqueous hydrochloric acid, but alcoholic hydrochloric acid yields leuco-A. → cyanidin.

Peat and miscellaneous. Alcoholic hydrochloric acid acting on peat produces cyanidin, doubtless by decomposition of leuco-A. Even a fibrous lignite gave indications of the presence of leuco-anthocyanin. Boxwood, alderwood, elm wood and teak were found to contain a little leuco-A. Richer are the wood of *Rhamnus frangula*, the seeds and stem of *Phoenix dactylifera* (date), seeds of *Lupinus polyphyllus*, of *Citrus aurantium*, of *C. nobilis Tangerana* and of *C. medica Limonum*. Leuco-anthocyanin also occurs in the grain of barley and oats.

Peltogyne pubescens and *P. porphyrocardia*, also a third unnamed "purpleheart," gave exactly the same results.

By extraction with cold 1 % hydrochloric acid some free anthocyanidin was obtained, and this was increased on boiling. Normally this was not separated and the leuco-A. was decomposed by boiling 10 % hydrochloric acid. The resulting anthocyanidin exhibits characteristic properties distinguishing it from all known substances of a similar type; it is therefore proposed to name it *peltogynidin*. Solutions of *peltogynidin* salts have a particularly bright cyanidin-like bluish-red colour and the Fe+ reaction is bright blue. The solution in concentrated sulphuric acid fluoresces green (resemblance to fisetinidin), but it has a pink colour (resemblance to cyanidin; distinction from fisetinidin). Stability in the oxidation test resembles that of cyanidin. Sodium acetate added to the amyl alcoholic solution gives a much redder shade than cyanidin, but in aqueous sodium carbonate the colour is pure blue. Its distribution to the delphinidin reagent is very high (100) and to the cyanidin reagent it is low (< 5). Ethyl acetate extracts it from a solution containing salt. In all these properties peltogynidin resembles very closely 5-*O*-methylcyanidin (VI). There is, however, one difference and that is the curious point that peltogynidin is not completely extracted by amyl alcohol from 1 % aqueous hydrochloric acid. Conversely continued washing of an amyl alcoholic solution with the aqueous acid gradually removes the colouring matter. This is such a constant property that we cannot attribute it to the effects of foreign substances. It is not due to insufficient acid hydrolysis, and if it is the result of acylation the group in question is not removed by boiling the solution after the addition of sodium hydroxide and subsequent addition of hydrochloric acid. This process is best accomplished by the use of the leuco-A. extracted by means of methyl alcoholic hydrogen chloride.

It is almost certain that peltogynidin is either a 5- or a 7-*O*-substituted-cyanidin; there may be one or more hydroxyl groups in the substituent.

The sapwood of *Peltogyne* spp. gave much the same results as the heartwood, and in both cases, as with *Eucalyptus marginata*, the leuco-A. was obtained in part in a solid form, capable of yielding peltogynidin on further decomposition. After extraction with aqueous acid is completed, further amounts of pigment can be obtained by the use of alcoholic hydrogen chloride.

Phaseolus multiflorus. The seeds of the scarlet runner bean "Champion" contain leuco-A. → cyanidin. The substance is concentrated in the pigmented seed-coat.

Pinus sp. A soft pine wood contained leuco-A. → cyanidin.

Polystichum aculeatum. The sporangia of the prickly shield fern gave leuco-A. → cyanidin. The roots and the epidermal layer of the young fern gave similar results.

Prunus Amygdalus. The endocarp contains leuco-A. → cyanidin.

P. communis, cortex and heartwood, contain leuco-A. → cyanidin and a much larger proportion of anthocyanidin tallying with 6-hydroxycyanidin. The cyanidin was removed by extraction with *cyclo*hexanol-toluene and separately examined.

P. spinosa (blackthorn), heartwood, gave leuco-A. → supposed 6-hydroxycyanidin. Again we do not claim to have identified the new anthocyanidin, but it is curious that the properties are in such close agreement with those of the proposed model. The sapwood gave leuco-A. → cyanidin.

Pterocarpus tinctorius, *Pt. soyeuxii* and *Pt. Osun*. These woods are closely related to the dyewood "Sanderswood" (*Pt. santalinus*) and doubtless owe their red colour to a pigment of the santalin type. We have not observed the presence of leuco-anthocyanin of the usual kind, but noted that the red aqueous acid extracts yield a yellow, green fluorescent, solution on shaking with ethyl acetate. On the addition of light petroleum the yellow pigment passes back into the aqueous

layer and with a red colour. This behaviour is reminiscent of some substituted 4-phenylflavylium salts which we happen to have had in our hands, and with the help of a carboxyl group and due regard to the work of A. G. Perkin [1899] and Cain and Simonsen [1912; 1914] on santalin, it is not difficult to construct a plausible constitutional formula for the pigment of the species of *Pterocarpus* mentioned above.

Pyrus malus. The heartwood gave leuco-A. → cyanidin. The seed-coats (Jonathan apple) are rich in leuco-A. → cyanidin; extraction with alcoholic hydrogen chloride was necessary.

Quercus pendunculata. The pericarps (acorns) contain leuco-A. → cyanidin.

Q. suber. Quite a large amount of leuco-A. → cyanidin can be obtained from ordinary corks, especially on treatment with alcoholic hydrogen chloride; hence the familiar discoloration.

Rosa. The outer red parts of the haws of roses do not yield leuco-A. to boiling aqueous hydrochloric acid, but the substance is present and can be extracted by alcoholic hydrochloric acid; the final anthocyanidin is cyanidin. The colourless inner portions afford more readily leuco-A. → cyanidin.

Scolopendrium vulgare. The sporangia gave leuco-A. → delphinidin.

Taxus baccata. The bark and heartwood of the yew were best worked up by means of alcohol. The leuco-A. gives an anthocyanidin closely resembling 6-hydroxycyanidin in its properties.

Trachylobium Hornemanianum. This wood gave the same results as *Peltogyne* spp. and *Copaifera pubiflora*.

Typha latifolia. The seeds on the spike of the reed-mace and also the stem gave leuco-A. → cyanidin.

Viburnum tinus laurustinus. The wood gave leuco-A. → cyanidin. Possibly some methyl ethers are also present because the distribution to the delphinidin reagent was somewhat too high for pure cyanidin.

Vitis vinifera. The green leaves of the Corinth vine gave leuco-A. → cyanidin and the colourless flesh of Alicante grapes gave the same results. The colourless flesh and skins of Almeria grapes were best worked up by means of alcoholic hydrogen chloride and ultimately afforded cyanidin. The coating of the seeds was found to be rich in its content of leuco-A. → cyanidin.

The description given by Rosenheim [1920] of his colouring matter from young vine leaves does not support the suggestion that the pigment was a pure anthocyanidin, and it was more probably a mixture with quercetin or quercitrin [Neubauer, 1873], such mixtures being extremely difficult to resolve by crystallisation from aqueous hydrochloric acid. The view that free anthocyanidin exists in the leaves is also not rigidly proved because it rested mainly on the high distribution to amyl alcohol. Owing to the courtesy of Dr Rosenheim we have been able to examine a small specimen of the pigment crystallised along with much flavonol derivative. It is completely extracted from aqueous solution by amyl alcohol like an anthocyanidin, but it does not pass from 1 % hydrochloric acid into ethereal picric acid. After successive hydrolysis with sodium hydroxide and hydrochloric acid a true anthocyanidin is recoverable, and this is completely extracted from watery solutions by means of ethereal picric acid. It appears therefore that the pigment of young vine leaves is a complex anthocyanin, but the quantity was insufficient for complete identification of the underlying anthocyanidin.

Synthesis of rhamnetinidin chloride (V). *ω*-3 : 4-Trihydroxyacetophenone (0·12 g.) was dissolved in the minimum of boiling ethyl acetate, the solution cooled and, after the introduction of 4-methoxy-2 : 6-dihydroxybenzaldehyde (0·10 g.), saturated with hydrogen chloride during 1·5 hours. A part of the salt crystallised and the remainder was obtained as picrate by washing the solution with water and adding saturated picric acid to the aqueous layer. The picrate was collected, added to the crystallised chloride and the whole dissolved in boiling alcohol containing a little hydrogen chloride. The salt was precipitated by means of ether, collected and crystallised by slow evaporation of a solution in

aqueous alcoholic hydrochloric acid as described by Robertson and Robinson [1927]. The *chloride* had all the properties mentioned by these authors as characteristic of rhamnetinidin chloride, and a direct comparison of colour reactions and distribution ratios showed that the salts were indeed identical. In addition to the properties already described rhamnetinidin is very largely extracted by the delphinidin reagent, wholly from dilute solutions; and it is more readily extracted by the cyanidin reagent than is cyanidin, but not so readily as peonidin. In sulphuric acid rhamnetinidin gives a pink solution with a faint green fluorescence.

Synthesis of 5-O-methylcyanidin chloride (VI). This salt was prepared like the foregoing, but using 2-methoxy-4 : 6-dihydroxybenzaldehyde in place of the isomeride; the whole salt (which did not crystallise), was converted into picrate and this derivative was crystallised several times from half-saturated aqueous picric acid containing a few drops of alcohol. The orange-brown, slender clustered needles (found in material dried at 100°: C, 50·2; H, 2·9; N, 7·9. $C_{22}H_{15}O_{13}N_3$ requires C, 49·9; H, 2·8; N, 7·9 %) dissolve in sulphuric acid to a pink solution exhibiting an intense green fluorescence. The chloride prepared in the usual manner is very similar to rhamnetinidin chloride, the only distinction being that the colour in aqueous sodium carbonate is greenish blue (rhamnetinidin-blue without green tinge) and the green fluorescence in sulphuric acid solution is much the more intense in the case of the 5-methyl ether.

Undoubtedly peltogynidin resembles cyanidin 5-methyl ether more closely than it does the 7-methyl ether; it should be noted that attachment of a substituent to positions 3, 3′, or 4′ is excluded.

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XXXII. A CONSTANT-VOLUME DIFFERENTIAL MANOMETER.

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For the gasometric study of physiological reactions, two types of manometer have been used, simple and differential: the simple manometer generally used is the blood-gas manometer of Haldane and Barcroft [1902], the instrument adopted by Warburg; whilst the differential principle is widely used in this country in the Barcroft apparatus [Barcroft, 1908], the application of which to the study of tissue metabolism has been described by Dixon and Elliott [1930]. In our experience the simple manometer is accurate and convenient for nearly all purposes. It is more economical in vessels than the differential manometer, in which each reaction vessel has its own compensation vessel, for several simple manometers can be used with a single thermo-barometer. Further, when a "blank" determination has to be made simultaneously with the main experiment, it is preferable to make it separately in a simple manometer rather than in the compensation vessel of a differential manometer. On the other hand, the simple manometer has certain limitations. Atmospheric pressure fluctuations may be so violent that the use of a separate thermo-barometer does not afford sufficient compensation, although we have never encountered such conditions in London. Again, the use of the simple manometer is restricted in cases where it is necessary to measure small differences between two large amounts of evolved or absorbed gas. For example, in the measurement of respiratory quotients in bicarbonate media [Dickens and Šimer, 1931], it was necessary to use a liquid of sp. gr. 4 as manometer fluid. It is obvious that under these conditions the amounts of gas which can be usefully evolved are limited by the length of the manometer limb, and that the sensitivity of the instrument is decreased by the use of a dense manometer fluid. If, however, the two amounts of gas, the difference between which is to be measured, be released simultaneously one in each vessel of a differential manometer, the amounts which can be released are no longer limited by the density of the manometer fluid, and this fluid can be chosen to suit the differences between the amounts and not the amounts themselves.

In the calculation of the gaseous exchange from the pressure readings, the Haldane-Barcroft manometer, as a constant-volume instrument, has the advantage of great simplicity. The pressure changes due to the evolution or absorption of a given gas have merely to be multiplied by a "vessel-constant" [Warburg, 1926], depending on the volumes of the vessel and of its contents and the solubility of the gas, in order to convert them accurately into mm.^{3} of gas at N.T.P. The Barcroft differential manometer, on the other hand, works neither at constant pressure nor at constant volume. The menisci in both limbs move, and it is necessary to use a rather complicated formula [Warburg, 1926] to

calculate the gaseous exchange from the observed pressure change. It was with the idea of enabling the simple, accurate, vessel-constant method of calculation to be used with the differential manometer that the constant-volume differential manometer described in this paper has been evolved.

Principle of the constant-volume differential manometer.

This manometer differs from those previously described in that both vessels are kept at constant volume. Each vessel is attached rigidly to its manometer limb, but each limb can be moved up and down independently of the other, as the lower ends are connected only by a length of rubber pressure-tubing filled with manometer fluid (Fig. 1). By raising or lowering one vessel with respect to

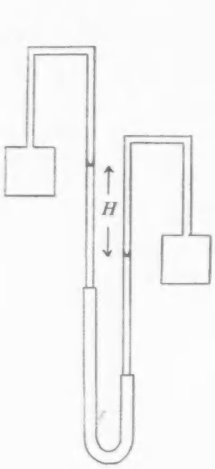

Fig. 1.

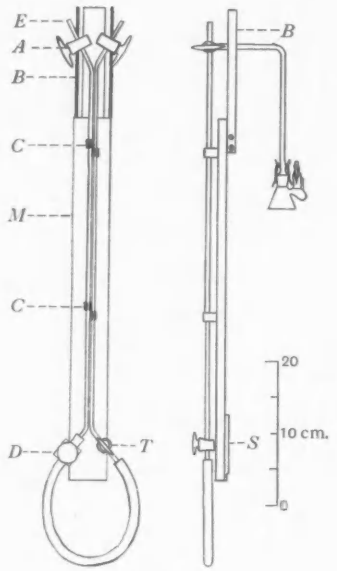

Fig. 2.

the other, the meniscus in each limb is kept at a given mark on that limb, so that each vessel is kept at constant volume. The difference in pressure between the two vessels is then given in terms of height of manometer fluid by the vertical distance between the marks on the two limbs.

In order to illustrate the method of calculation used, two cases will be discussed.

I. One vessel employed for temperature compensation only. If the vertical distances between the menisci, adjusted to their respective marks, be respectively H_0 and H_1 before and after the evolution of a given amount of a gas in the reaction vessel, then the observed pressure change $h = H_1 - H_0$ is related to the volume x mm.^{3} occupied by that amount of gas at N.T.P. by

$$x = k'_g.h \qquad \text{.....(1),}$$

where k_g^t is the vessel-constant of the reaction vessel for the particular gas under the experimental conditions, and is given by

$$k_g^t = \frac{v_F \alpha_g^t + v_G \cdot \frac{273}{t+273}}{P_0},$$

where v_F = mm.^{3} liquid in reaction vessel.
v_G = mm.^{3} gas in reaction vessel and manometer tubing as far as mark.
α_g^t = Bunsen absorption coefficient of the gas at $t°$.
P_0 = pressure of one atmosphere expressed in mm. of manometer fluid (for Brodie fluid P_0 is approximately 10,000).

II. Different amounts of the same gas are released simultaneously in both vessels. It is arranged that both vessels shall have the same vessel-constant k_g^t for that gas: methods of ensuring this are described later. If the amounts of gas evolved in vessels A and B would occupy volumes x_A and x_B respectively at N.T.P., and if as before h denotes the observed pressure change, then

$$\pm (x_A - x_B) = k_g^t . h \qquad \ldots\ldots(2).$$

Construction.

The instrument is shown in Fig. 2. Each vessel is attached by a ground joint to its manometer. The manometer with attached vessel can be raised or lowered as required, the manometer limb sliding in the spring clips C, and the horizontal tubes being kept at right-angles to the mounting board M by the vertical slots formed by the guides B and the wooden centre-piece. The board M has the brass sleeve S for fixing on to the usual Warburg apparatus by which the vessels are shaken in the well-stirred thermostat (depth 40 cm.). The taps A are T-bored so that the vessels, the manometer limbs and the outside air may be connected in pairs or together. Brodie fluid is used for the manometer liquid, which may be prevented from moving by closing the tap T. The level of the fluid in the manometer limbs is adjusted by means of the screw-clip D which compresses the rubber tubing; metal plates are soldered on to the clip so as to increase the area over which the rubber is compressed. If the rubber were inextensible, it would not be necessary to alter this adjustment once it had been made, but in practice a slight re-adjustment of the screw-clip is necessary for every reading.

The graduations are etched on the glass limbs. One limb (the left) bears a single ring at its mid-point, to which the level of manometer fluid is always adjusted. The other limb carries a scale of mm., 0 to 300, reading from top to bottom, the fluid level in the limb being kept at the 150 mark at the mid-point of the limb. It is important that the limbs should be almost touching, and the scale on the inner side of the capillary; it is then possible to read accurately on the graduated scale on the one limb the level of the ring on the other. A single reading thus determines the relative positions of the two menisci; and the difference between this reading and 150 gives the difference in pressure between the two vessels, expressed in mm. of manometer fluid. The procedure in taking a series of pressure readings is thus exactly the same as with the simple manometer, with the added advantage that no thermo-barometer corrections are necessary.

One or two points in the construction are to be noted. During the raising and lowering, whilst the vessels always remain completely immersed in the thermostat, varying lengths of capillary tube are exposed. It is therefore necessary that the amount of air in the part of the capillary tube involved shall be so small that the change of pressure caused when it is moved up and down is negligible. In order to give a sufficient margin of safety even when the readings involve the full

displacement of the scale, the whole of the tube between the vessels and the tap *A* is made of capillary tubing of such a bore that the increase of pressure when the air in the vertical portion is raised from room temperature to 38° is less than 1 mm. Brodie fluid. It was necessary to use tubing of 0·3 mm. bore, and this was found to be perfectly satisfactory in practice. The manometer limbs are of 1 mm. capillary tubing.

The vessels used may be of any of the usual types [see for example Krebs 1928]; for filling with gas mixtures of known composition the stoppers should be of the type described by Warburg and Kubowitz [1929]. We would emphasise the importance of making the ground joints really tight before the beginning of the experiment by working them in until they will no longer turn. Accidental displacement of the stoppers before this is done is prevented by rubber bands stretched over hooks on the stoppers (Fig. 2).

It should also be noted that either limb with its vessel may be detached separately from the board and removed from the thermostat; this enables fluid to be tipped from bulbs into the vessels one at a time. In this flexibility it has a distinct advantage over the Barcroft manometer. Either limb may be opened to the air by the T-tap; the instrument may then be used as a simple manometer in conjunction with a thermo-barometer. The taps also enable either vessel to be completely shut off: this has an important application, as will be seen later.

The apparatus is described here in its simplest form; it is evident that rack and pinion adjustment of the manometer limbs or similar mechanical additions could be fitted.

Method of use.

The vessels are filled with a suitable gas by connecting the leading tubes by means of a T-piece to the tubes *E* of the manometers. It is desirable to pass the gas under rather higher pressure than usual owing to the narrow capillary tubes. The arrangement for filling the manometer with gas is shown in Fig. 3. The

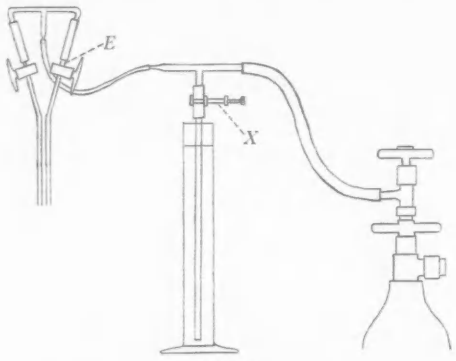

Fig. 3.

pressure from the gas cylinder is increased until gas bubbles pass gently through the water in the safety device. The clip *X* is then screwed up. When enough gas has passed, *X* is unscrewed, the stoppers in the manometer vessels closed, the manometer taps turned and the gas supply disconnected. This filling with gas may be done with the apparatus in or out of the thermostat. After this stage the carrying out of the experiment calls for no special comment.

Calibration. The vessels and capillaries as far as the mark are calibrated by filling with mercury. The vessels are first filled and then slowly pushed on to the ground joint. Owing to the fineness of the capillaries it will be found convenient either to apply suction at *E* while the vessel is being attached, or to warm the vessel slightly after it has been attached, and so to link up the mercury with a thread in the wide capillary.

The vessels should be made of nearly the same size; several methods are then available for securing equality of vessel volumes. A glass bead of suitable volume may be added to the larger vessel, but a more satisfactory permanent adjustment is easily secured by alteration of the size of the side-bulb by blowing, followed by suitable grinding in of the ground joints as a fine adjustment. A certain latitude is permissible; for example, with two 26 cc. vessels containing 3 cc. liquid a difference in vessel volume of 0·1 cc. causes a difference of level of only 2 mm. of Brodie fluid when 1000 mm.^{3} CO_2 at 30° are evolved in both vessels. A correction to allow for this slight change may readily be made, and since the correction is proportional to the total pressure change for ordinary releases of the order of 200 mm.^{3} the correction will lie within the limits of error of reading.

The equality of the vessels is conveniently checked by this method by the liberation of CO_2 from a measured amount of $NaHCO_3$ by means of excess acid. 3 cc. of a 0·2 % solution of $NaHCO_3$ previously saturated with the gas mixture are weighed into each of the clean dry vessels. The acid (0·4 cc. *N* HCl) is pipetted into the side-bulbs and the vessels are connected with their manometers and filled with oxygen or nitrogen containing 5 % CO_2. This amount of $NaHCO_3$ liberates some 1600 mm.^{3} CO_2 at N.T.P. Any change of pressure on acidification of both vessels is noted, and may be applied as a correction in the way already mentioned.

As a further control on the use of the instrument we have liberated known small amounts of CO_2 by adding measured amounts of standard acid (0·2 cc. 1·02 *N*/20 H_2SO_4, volume checked by weighing) from the side-bulbs to an excess of the bicarbonate solution (1·5 cc.) contained in the main part of the vessels, the gas space being filled with a gas mixture containing 5 % CO_2. This method is convenient for controlling the accuracy of calibration and for demonstrating that no measurable error is introduced by the raising and lowering of the fine capillaries in the thermostat (temp. 38°). For the latter purpose care was taken to arrange the menisci at the beginning of the experiment at very different levels; the acid was then rinsed into the bicarbonate in the vessel occupying the highest position and after time had elapsed for temperature equilibrium a reading was taken. The acid and bicarbonate were then mixed in the other vessel, when the level regained its former value. This was repeated many times, first one vessel then the other being at the highest point of its travel at the beginning of the experiment. The results of one such series are shown below together with a simultaneously made series using the simple Warburg manometer. Volume of CO_2 in mm.^{3} at N.T.P. evolved from 0·2 cc. 1·02 *N*/20 H_2SO_4 (calculated CO_2 = 227 mm.^{3}):

Simple manometer: 225, 226·5, 231, 227·5, 226, 225, 231, 227·5, 225, 226·5, 226, 229·5, 226, 228·5, 226, 227. *Mean:* 227 (highest 231, lowest 225).

Differential manometer: 224·5, 224·5, 225·5, 226·5, 226, 227·5, 228, 227·5, 228, 229·5. *Mean:* 227 (highest 229·5, lowest 224·5).

The satisfactory agreement shows that the error is little greater than that of the scale reading ($\pm$ 0·5 mm.), and that the submerged length of capillary in the differential manometer introduces no measurable error.

In order to indicate the possibilities of the instrument, we describe an

experiment in which the anaerobic metabolism of rat testis was studied. Pieces of tissue, of equal wet weight, were placed in 3 cc. of glucose bicarbonate Ringer in each vessel of the manometer, a gas mixture containing 5 % carbon dioxide in nitrogen was passed for 4 minutes, and the apparatus shaken in the thermostat at 38° for 10 minutes. The pressure in the vessels was allowed to fall to atmospheric; the vessels were then closed again to the atmosphere and connected to the manometer limbs. An exact reading was taken; the right-hand tap was then turned so that the vessel was shut off and the limb opened to the air. An excess of acid was tipped from a bulb into the closed right-hand vessel. The evolution of gas in the left-hand vessel was followed, using the apparatus as a simple manometer in conjunction with a separate thermo-barometer. After 90 minutes, 226 mm.^{3} of carbon dioxide had been evolved. The meniscus was then brought to the 150 mark on the right-hand limb, the tap *T* was closed, and the right-hand vessel was connected to the limb by turning the tap *A*. Excess acid was tipped from the bulb into the left-hand vessel. After a few minutes the tap *T* was opened, so that the Brodie fluid now showed the pressure difference between the two vessels. After 10 minutes this became constant. Provided that the tissue had itself produced or consumed no carbon dioxide, so that the total CO_2 in the vessels remained unchanged, the final reading should be the same as the initial reading. As will be seen from the protocol, within the errors of reading, this was indeed so.

Protocol.

Ringer solution: 0·2 % glucose. Bicarbonate content = 580 mm.^{3} per cc.

Vessels: 3 cc. Ringer in main part. 0·5 cc. 0·5 *N* HCl in bulb; $k^{38}_{CO_2} = 2{\cdot}20$.

Tissue: Rat testis. Rinsed 5 mins. in Ringer and afterwards in two changes of salt solution. 150 mg. wet weight placed in each vessel. Dry weights 11·9 mg. and 12·5 mg.

Time: 0 min. Initial reading 148·5. Right-hand vessel closed and limb opened to air; contents of vessel acidified.

Time mins.	Reading	*h* (corrected for thermo-barometer)
1	146·5	0
20	174	29·5
40	197·5	53
60	220·5	74·5
90	251	102·5

CO_2 evolved = 102·5 × 2·20 = 226 mm.^{3} $Q^{N_2}_M = 12{\cdot}5$. Right-hand meniscus adjusted to 150 mark. Tap *T* closed. Right-hand vessel connected to limb. Acidify left-hand vessel contents. Tap *T* opened at 94 mins.

Time mins.	Final reading
100	144·5
110	144·5

Difference between final and initial level	= −4 mm.	corrected pressure change = −0·5 mm.
Correction for pressure change on acidifying 3 cc. Ringer in each vessel of same apparatus	= −3·5 mm.	

We are at present developing an improved method for the measurement of respiratory quotient, respiration and glycolysis of tissues in bicarbonate media using the manometer here described.

SUMMARY.

A differential manometer is described in which both vessels are maintained throughout at constant volume.

The pressure change is given as the difference of single scale readings, and the corresponding gas volume is derived from this pressure change by multiplication by a simple vessel constant.

The instrument thus combines the differential manometer's advantage of compensation with the simple manometer's simplicity of reading and calculation.

One of us (F. D.) is a member of the scientific staff of the Medical Research Council, and the other (G. D. G.) wishes to make acknowledgments to the Halley-Stewart Trust Fund for a scholarship.

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XXXIII. THE ALLEGED ANTISCORBUTIC ACTIVITY OF A MIXTURE OF METHYL-*nor*NARCOTINE AND GLYCURONIC ACID.

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(*Received December 31st, 1932.*)

THE experiments to be described in this paper were undertaken in order to appraise the claim made by Rygh and Rygh [1932, 2] that a mixture of methyl-*nor*narcotine and glycuronic acid possesses antiscorbutic activity. In a preliminary communication [Dann, 1933] it was stated that the experimental observations made by Rygh and Rygh could not be confirmed, but a detailed description of the work performed is presented here for the first time. It will be recalled that Rygh [Rygh, Rygh and Laland, 1932; Rygh and Rygh, 1932, 1] at first put forward the claim that synthetic methyl*nor*narcotine itself is identical with vitamin C, a claim which has been widely acknowledged, although it is based on observations which other workers have been unable to repeat [Smith and Zilva, 1932; Grant, Smith and Zilva, 1932; Dalmer and Moll, 1932; Dann *et al.*, 1932]. The many criticisms to which Rygh's claims are open will not be dealt with here as they have already been summarised by Harris [1932]. For the present purpose it is only necessary to note that Rygh based his conclusion that methyl*nor*narcotine and vitamin C are identical upon (*a*) the assumption that vitamin C is formed by the action of ultra-violet radiations during the ripening of fruit; (*b*) the allegation that unripe oranges contain narcotine which disappears during ripening; and (*c*) the allegation that synthetic methyl*nor*narcotine prepared from narcotine by irradiation or hydrolysis will protect guinea-pigs fed on a vitamin C-free diet from scurvy, although they decline in weight and die from some other (undetermined) cause as rapidly as the animals receiving no addition to the basal diet. With regard to these bases of Rygh's hypothesis: (*a*) Eggleton and Harris [1925] showed that the synthesis of vitamin C by plants is independent of ultra-violet radiations; (*b*) Dalmer and Moll [1932] and Grant, Smith and Zilva [1932] have been unable to find narcotine in the juice of unripe oranges, although added narcotine was readily recovered; and (*c*) irradiated narcotine and synthetic methyl*nor*narcotine prepared by the hydrolysis of narcotine according to the directions of Rygh and Rygh [1932, 1] are found by Smith and Zilva [1932], Grant, Smith and Zilva [1932], Dalmer and Moll [1932], and Dann *et al.* [1932] to be devoid of antiscorbutic activity. It has also been shown by Dalmer and Moll [1932] that the so-called methyl*nor*narcotine of Rygh is in reality a mixture.

Rygh has met these criticisms by an elaboration of his earlier views [Rygh and Rygh, 1932, 2], without bringing forward any further evidence to uphold the correctness of the observations which have been disputed. He has definitely embraced the theory of the complex composition of vitamin C, suggesting that one component is methyl*nor*narcotine and that any uronic acid may act as the

[1] Medical Research Scholar of the Worshipful Company of Grocers.

other component. Thus, according to Rygh, the anti-scorbutic activity of Szent-Györgyi's hexuronic acid is due to contamination of the hexuronic acid with small amounts of methyl*nor*narcotine, which gives a mixture capable of replacing vitamin C. To account for the lack of antiscorbutic activity of preparations of synthetic methyl*nor*narcotine prepared by other workers, he has suggested that his earlier method of hydrolysing narcotine was too drastic and was liable to hydrolyse further the methyl*nor*narcotine at first produced; and he has announced that the irradiation of narcotine does not give rise to a mixture possessing anti-scorbutic activity if the narcotine is kept cool. A milder hydrolysis of narcotine is described, which yields a product designated "30 % methyl*nor*narcotine."

The only experimental observations offered in support of these hypotheses are given in the following words: "Vier Meerschweinchen desselben Wurfes wurden auf C-vitaminfreie Kost gesetzt. Zur Verwendung kam die modifizierte Göthlinsche Grundnahrung, deren Zusammensetzung aus unserem vorigen Aufsatz ersichtlich ist. Zwei von den Tieren erhielten täglich ½ mg. Glucuronsäure. Diese zwei Tiere verloren nach 14 Tagen stark an Gewicht und gingen nach etwa 4 Wochen an schwerem Skorbut ein. Die zwei anderen Tiere erhielten ebenso ½ mg. Glucuronsäure, dazu noch 10γ 30 % iges Methylnornarkotin, beides täglich. Die Tiere lebten die ganze Versuchszeit (50 Tage) durch, bei bestem Gesundheitszustand, nahmen dauernd an Gewicht kräftig zu. Als sie bei Versuchsschluss zerlegt wurden, ergaben sie makroskopisch völlige Skorbutfreiheit."

In the following section an account is given of the writer's experience in feeding methyl*nor*narcotine and glycuronic acid to guinea-pigs.

Experimental.

Arrangement of the feeding test. Young male guinea-pigs weighing 250–300 g. were placed upon the usual scurvy-producing diet used in this laboratory (bran 80 parts, oats 720, egg-yolk 40, salts 84, cod-liver oil 1 %). During a preliminary period each animal received a daily supplement of 10 g. of cabbage in order to maintain normal health until it became accustomed to the new diet. At the end of 7 days all the guinea-pigs had commenced to grow rapidly on the new regimen, and the experimental period proper commenced. The animals were divided into five groups as evenly matched as possible, three in each group, and the different groups received the basal diet and tap-water *ad lib.* together with the following daily supplements. Group 1 (negative controls) received no supplement. Group 2 (positive controls) each received 10 g. cabbage. Group 3, each received 0·5 mg. glycuronolactone. Group 4, each received 0·5 mg. glycuronolactone + 10γ "30 % methyl*nor*narcotine." Group 5, each received 10γ "30 % methyl*nor*narcotine."

The supplements were administered daily in aqueous solution. Fresh solutions were made each day, the materials for each dose being dissolved in distilled water in such amounts that the requisite dose of solution for each group was 0·5 cc., which was pipetted into the animal's mouth. The specimen of "30 % methyl*nor*narcotine" used was kindly supplied by Dr Rygh and was a light brown coloured solid, which melted sharply at 218° (uncorr.) with decomposition. Glycuronic acid was used in the form of its lactone, of which a specimen was kindly given by Dr E. L. Hirst.

Examination of the guinea-pigs for scurvy. The animals were weighed at intervals and the growth curves are reproduced in Fig. 1. After 16 days of the experimental period, all the positive control animals were still growing steadily,

while all the remainder (except one which had died after 10 days) were losing weight rapidly. All the survivors were killed on the 16th day and at the autopsy on each animal the knee joints were examined for signs of haemorrhage, the ribs were examined for signs of beading at the costo-chondral junctions and the lower jaws were cut away for transverse sections of the roots of the incisors to be made in the usual way[1]. On examination of the tooth sections, the degree of protection from scurvy afforded to each animal was evaluated according to the scale devised by Key and Elphick [1931]. The results of all examinations of joints, ribs and teeth are recorded in Table I. From these data it is concluded that all the

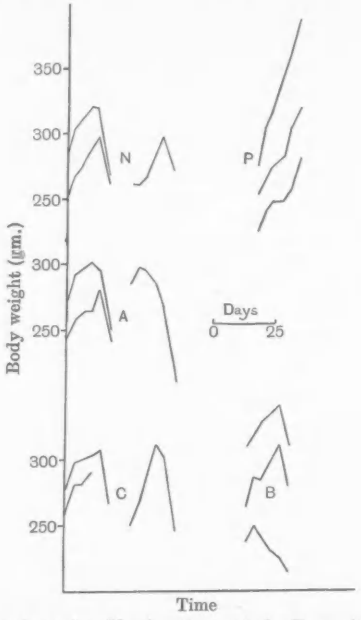

Fig. 1. Growth curves of guinea-pigs. N—negative controls. P—positive controls. A—animals receiving 0·5 mg. glycuronolactone daily. B—animals receiving 0·5 mg. glycuronolactone + 10γ "30 % methyl*nor*narcotine" daily. C—animals receiving 10γ "30 % methyl*nor*narcotine" daily.

animals which received supplements of glycuronic acid, methyl*nor*narcotine, or a mixture of the two, developed scurvy as severely as the negative control animals while the positive controls remained free from scurvy. Similar results were obtained with a second specimen of "30 % methyl*nor*narcotine," prepared in this laboratory according to the instructions given by Rygh and Rygh [1932, 2].

Discussion.

It has proved impossible, by the methods used in this laboratory, to detect any antiscorbutic activity in a mixture of glycuronic acid and methyl*nor*narcotine in the proportions and quantity stated by Rygh and Rygh [1932, 2] to support good growth in guinea-pigs and to protect them completely from scurvy when

[1] I wish to thanks Messrs Hughes and Cox of the Department of Animal Pathology, Cambridge University, who carried out the preparation of the tooth sections.

Table I. *Examination of the experimental animals for signs of scurvy.*

Guinea-pig	Daily addition to basal diet	State of growth after 16 days	Haemorrhage of knee joints	Beading of ribs	Degree of protection from scurvy shown by the teeth
1	None	Losing weight rapidly	+ +	+ +	1
2	,,	,,	+	+	0
3	,,	,,	+ +	+	0
4	0·5 mg. glycuronolactone	,,	+ +	+ +	1
5	,,	,,	+	+	0
6	,,	,,	+ +	+ +	0–1
7	0·5 mg. glycuronolactone + 10γ "30 % methyl-*nor*narcotine"	,,	+	+	1
8	,,	,,	+ +	+ +	0
9	,,	,,	+	+ +	0
10	10γ "30 %" methyl*nor*-narcotine	,,	+	+	0
11*	,,	—	—	—	1
12	,,	Losing weight rapidly	+ +	+	0
13	10 g. cabbage	Growing well	0	0	4
14	,,	,,	0	0	4
15	,,	,,	0	0	4

Explanation of symbols.

Column 4. 0 signifies no haemorrhage.
+ signifies slight haemorrhage.
+ + signifies severe haemorrhage.

Column 5. 0 signifies no rib-beading.
+ signifies slight rib-beading.
+ + signifies very marked rib-beading.

Column 6. The figures correspond to the scale drawn up by Key and Elphick, 0 indicating no protection, and 4 indicating complete protection from scurvy.

* Guinea-pig No. 11 died on the tenth day of the experiment, and no examination of the joints or ribs took place.

fed on a scorbutic diet for a period of 50 days. The test was carried out under the same conditions as the test of hexuronic acid reported from this laboratory by Harris, Mills and Innes [1932] when it was found that degree of protection from scurvy No. 4 on Key and Elphick's scale was given by hexuronic acid in a daily dose of 2 mg.

The contentions of Rygh and Rygh [1932, 2] (*a*) that the antiscorbutic activity of hexuronic acid is due to contamination of the hexuronic acid by methyl*nor*narcotine which gives rise to a mixture capable of functioning as vitamin C, and (*b*) that the previously known uronic acids are interchangeable with Szent-Györgyi's hexuronic acid in animal metabolism, are based on the assumption that hexuronic acid is a true uronic acid belonging to the same family as glycuronic and galacturonic acids. When first he isolated hexuronic acid Szent-Györgyi [1928] supposed it to be the lactone of an acid isomeric with these two uronic acids, but it has recently been shown by Cox, Hirst and Reynolds [1932] that the structure of hexuronic acid is quite dissimilar from that of either glycuronic acid or its lactone. The likelihood that hexuronic acid and glycuronic acid play interchangeable or closely similar parts in metabolism is therefore much smaller than Rygh assumes.

Regarding the antiscorbutic activity of narcotine derivatives, it should be borne in mind that no experimental observation of Rygh's concerning these substances has gone undisputed by other workers, save the statement of Römcke and Rygh [1932] that methyl*nor*narcotine has served to cure scurvy in human patients. Details of one case only have been published so far as the writer is aware, and the patient was given methyl*nor*narcotine as a supplement to a diet containing milk, meat-cakes and fish. As pointed out by Harris [1932], the milk would almost certainly contain vitamin C, and possibly the meat-cakes also, so that the use of this basal diet is sufficient to destroy the value of the observation.

Summary.

A mixture of methyl*nor*narcotine and glycuronic acid has been found to be devoid of antiscorbutic activity.

Reasons are presented for believing that hexuronic acid does not owe its activity to methyl*nor*narcotine contaminating the acid, as claimed by Rygh.

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XXXIV. LARGE SCALE PREPARATIONS OF VITAMIN B_1 AND VITAMIN B_4 CONCENTRATES.

By HENRY WULFF KINNERSLEY, JOHN RICHARD O'BRIEN, RUDOLPH ALBERT PETERS and VERA READER.

From the Department of Biochemistry, Oxford.

(*Received January 1st, 1932.*)

In this communication, we are recording the details of a large scale process for preparing vitamin B_1 and B_4 concentrates, which has stood the test of over a year's working in this laboratory. It is based upon the original Kinnersley and Peters procedure, and has been briefly described [Peters *et al.*, 1931].

We shall describe the technique upon the basis of 100 kg. (2 cwt.)[1] of yeast, but it will be realised that economy in time results from running several processes together. We ourselves have worked up to the charcoal stage in the laboratory as much as 300 kg. (6 cwt.) of yeast in a week. 200 kg. is however more convenient with the staff and apparatus available. Limitation of scale depends more upon apparatus than increase of staff.

Baker's yeast (supplied by D.C.L. Company) has been used throughout and can be relied upon to give standard results. It is kept at room temperature for 1–2 days before use in the 7 lb. bags as supplied. Examination of our schedule of time and labour shows that our methods compare favourably with those of others, and we can see no inherent objection, apart from the expense, to working upon the 1000 kg. scale[2].

It may be stated that we have tried several variations on the procedures described. In our experience rather trifling changes, to which no theoretical objection can be seen, usually diminish the yield, and should not be embarked upon lightly. This is probably due to the complexity of the mixtures from which adsorption takes place.

Reagents required (100 kg. scale).

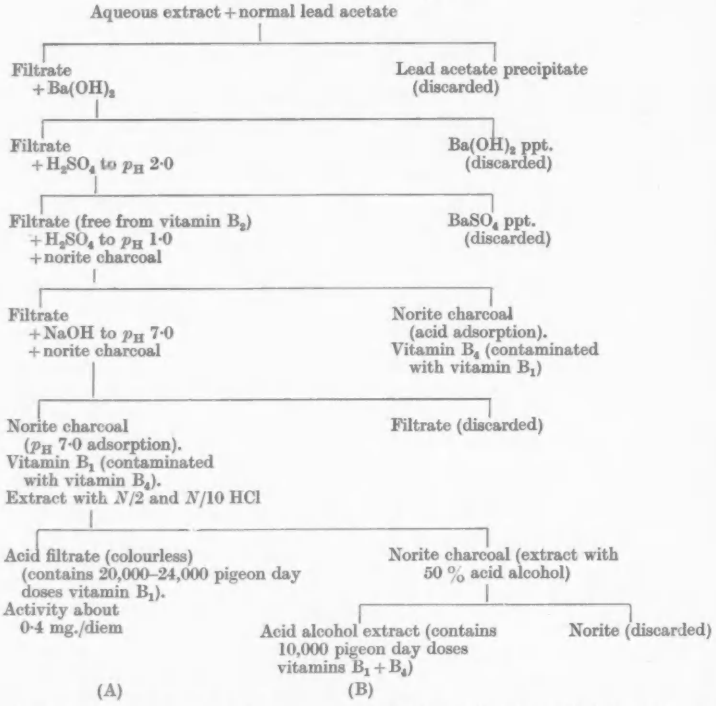
Lead acetate (normal)	2250 g.
$Ba(OH)_2, 8H_2O$	9–10 kg.
NaOH	1500 g.
H_2SO_4 (conc.)	1500–2000 cc.
Norite charcoal	2000 g.
HCl (conc.)	350–500 cc.
Alcohol	6–8 litres.

The yields are expressed in pigeon day doses. One such dose is approximately equivalent to 1 unit of vitamin B_1. (1 pigeon dose $\equiv$ 10 mg. Jansen acid clay.) Previously, we have stated 1 pigeon dose was equivalent to 15 mg. Jansen clay. Further work with doses of 50 mg. instead of 100 mg. has led to the revised figure.

[1] 2 cwt. = 101·5 kg. Round figures used in text for convenience.

[2] The general speed can be judged from the fact that vitamin B_1 crystals have been reached in 14 days from the initial boiling.

Plan. 100 kg. *baker's yeast.*


Aqueous extract + normal lead acetate
Filtrate + $Ba(OH)_2$
Lead acetate precipitate (discarded)
Filtrate + H_2SO_4 to p_H 2·0
$Ba(OH)_2$ ppt. (discarded)
Filtrate (free from vitamin B_2) + H_2SO_4 to p_H 1·0 + norite charcoal
$BaSO_4$ ppt. (discarded)
Filtrate + NaOH to p_H 7·0 + norite charcoal
Norite charcoal (acid adsorption). Vitamin B_4 (contaminated with vitamin B_1)
Norite charcoal (p_H 7·0 adsorption). Vitamin B_1 (contaminated with vitamin B_4). Extract with $N/2$ and $N/10$ HCl
Filtrate (discarded)
Acid filtrate (colourless) (contains 20,000–24,000 pigeon day doses vitamin B_1). Activity about 0·4 mg./diem
Norite charcoal (extract with 50 % acid alcohol)
Acid alcohol extract (contains 10,000 pigeon day doses vitamins $B_1 + B_4$)
Norite (discarded)
(A)
(B)

(A) Can be used to obtain crystalline preparations of vitamin B_1 as described in a separate communication (Kinnersley *et al.*, 1932; 1933).

(B) Is available as a basal source of vitamins B_1 and B_4 for various rat and pigeon tests or can be used for clinical purposes.

Preparation of aqueous yeast extract. 20 litres of tap-water are heated to boiling in a steam-heated pan (steam pressure 10 lb.). During the heating, 42 lb. (19 kg.) of yeast are crumbled by hand and stirred gradually into the water as it boils. The whole is made cool by the addition of the yeast and is raised to the boil again. It is then transferred by enamel pail to a sedimentation centrifuge. The one used for this purpose was supplied by Messrs Broadbent and has a rustless steel basket of diameter 20–22 in. Centrifuging for 15 minutes is usually sufficient, after which the clear yellow layer is removed by the skimmer: final temperature 50–60°. The moisture of the yeast residue varies somewhat at different times of the year. The volume of extract obtained from 100 kg. of yeast is 124–7 litres. In order to economise time, a second batch of water is heated during the centrifuging, which is then ready immediately for the second batch of 19 kg. of yeast. In all the extraction of the 100 kg. is complete in 5 batches, and occupies the time of one man for 7–8 hours. The extracts are allowed to cool to a temperature of 18° or under.

The extract (p_H 6·5–6·8) so obtained is allowed to stand overnight after addition of 16 cc. 25 % normal lead acetate per litre; making the p_H 5·0–6·0.

Notes. (1) Acetic acid (1 %) has been often recommended for the extraction, and is considered ideal upon theoretical grounds, in order to ensure coagulation of proteins, *etc.* We have found no improvement from its use.

(2) The "standing" period overnight seems to be essential; earlier attempts to dispense with it gave poor yields of vitamin B_1. It is not clear what happens, but some change (possibly autolytic) must liberate the vitamins in suitable form for adsorption.

(3) Originally two extracts of the yeast were made, but we now dispense with the second extraction. The slight gain in yield does not compensate for the time lost in making it.

Lead acetate precipitation. After standing for 17–20 hours, the yeast extract is treated with 25 % normal lead acetate until maximum precipitation has occurred. The tendency for fresh additions to produce a faint cloud marks the completion of this process; about 2000–2500 cc. are needed for the aqueous extract from 100 kg. The extracts are then run through a Sharples supercentrifuge (size No. 5 long boss bowl, revs. 16,000). The barrel requires one cleaning during this process, each 50 kg. equivalent running through in 10–15 minutes; nitrogen is passed through the centrifuge and solutions to minimise oxidation. As soon as the filtrate from the first 50 kg. is ready, it is treated with baryta, so that the processes run concurrently. The weight of wet lead acetate precipitate so obtained is approximately 5000–6000 g. It may be dried and stored if required. The lead acetate stage occupies 1–1½ hours.

Note. In the absence of a Sharples centrifuge, the lead precipitate is best filtered upon funnels with folded paper, and the filtration occupies some 24 hours.

Baryta precipitation. During the centrifuging or filtering of the lead precipitate, 1500 cc. beakers (or flasks) are set up each containing 200–300 cc. H_2O. Solid baryta is added until the mixture occupies about 2/3 the volume of the flask. The mixture is heated vigorously to boiling, and more baryta added until a thick suspension is obtained consisting of about 1200 g. baryta in 300 cc. H_2O.

To each 32 litres of filtrate from lead precipitate, 1000–1500 cc. of hot "baryta" are added with vigorous stirring; the final temperature should not exceed 25°. The vessels are allowed to stand for 3–5 minutes. If flocculation does not occur immediately, a few cc. of lead acetate solution may be added and the solution agitated. The mixture is filtered at once and should filter rapidly. A number of 6-inch funnels has been lately used complete with folded filter-papers. The funnels discharge into two rectangular gutters which collect the filtrate into a receptacle. As soon as possible, sulphuric acid is added to the filtrate until the reaction is acid to Congo red (or purple to thymol blue; about p_H 1·0). The latter acidity induces the more rapid sedimentation of the barium sulphate, and inhibits to some extent the frothing caused by the centrifuging. If the filtrate from baryta is not quickly acidified, considerable darkening in colour may take place; there is also risk of destruction of the vitamins. The lead acetate filtrate from 100 kg. can be taken through the baryta stage with the funnels in 4 hours by one worker.

Notes. Practice may be required to adjust the flocculation stage. If successful the filtration should be very rapid indeed (almost like water). Fractious extracts may be cured by a small addition (1–2 cc./litre) of lead acetate before addition of baryta.

Removal of $BaSO_4$. This can be allowed to settle, or is preferably removed by the Sharples centrifuge. The latter process only occupies 2 hours. It is usually run in 3 batches of 40–60 litres each.

Charcoal adsorptions.

Adjust p_H of the filtrate from $BaSO_4$ to 1·0 (slight purple to thymol blue).

Acid charcoal adsorption. To each batch of 32 litres[1], add 180 g. dry norite charcoal, purified previously by boiling with conc. HCl and washing till free from acid[2]. Allow to stand for 20 minutes, stirring frequently, before commencing filtration. Filter through 10 Büchner funnels (6 inch). Add the suspension continuously, collecting the filtrate as it appears and never letting the charcoal on the funnels become completely dry. The time of filtration varies, but usually one worker can filter the extract from 100 kg. (120 litres) in 2 hours through one set of filter-papers. The charcoals (700 g.) are washed with $N/10$ H_2SO_4, 200 cc. per Büchner funnel. The filter-papers and charcoals are transferred to a large beaker (4 litre) and treated with 800 cc. 50 % alcohol (by volume) and enough conc. HCl to bring to p_H 1·0. At this stage they may be left overnight. It is somewhat easier to handle the charcoal, if the filter-papers are extracted separately, but this is not essential.

Note. This step was introduced by one of us (V.R.) as a modification of the original shortened process of Kinnersley and Peters. The extra acidity is found to cause adsorption of much of the vitamin B_4 in a form suitable for subsequent separation from vitamin B_1 [see Barnes, O'Brien and Reader, 1932].

Charcoal adsorption at p_H *7·0.* To the charcoal filtrates from "acid charcoal" add 20 % NaOH, until the p_H is 7·0 $\pm$ 0·04. The amount varies to some extent, but usually lies between 1000 and 1200 cc./32 litres. It is important not to make much more alkaline than p_H 7·0. Even a temporary sojourn at p_H 7·6–8·0 is apt to effect some change and it is advisable to approach p_H 7·0 with caution. A precipitate sometimes appears, which is not removed separately. To each batch of 32 litres at p_H 7·0, add 320 g. norite charcoal, and stir well. Adsorption is rapid; we allow to stand 10 minutes, and then commence to filter through the 10 Büchner funnels, as before. The filtration is now more rapid and the filtrate practically colourless. The charcoal on the funnels is thoroughly washed with distilled water 3–4 times, some 1500 cc. to each Büchner funnel.

The 1200 g. charcoal (p_H 7·0) (with or without the filter-papers) is placed in a large vessel (6 litre), and 2 litres of cold $N/2$ HCl added. After thoroughly stirring, the p_H is tested with Congo red. If not acid, conc. HCl is added until the mixture turns Congo red blue. It may now be left overnight.

The stages from lead acetate to this point for a 100 kg. batch can be carried through in one working day by two workers, if care is taken to organise the work. As here arranged, with use of the supercentrifuge, parts of the following stages can be run concurrently, (1) lead acetate removal and filtration of baryta flocculate, (2) the latter and removal of $BaSO_4$, (3) removal of $BaSO_4$ and acid charcoal filtration.

Note. In the initial work care was taken to use charcoal which had been purified by extraction with conc. HCl, and subsequent washing until free from acid. Owing to the considerable labour involved, we have recently substituted commercial norite. The metals (mainly Zn and Fe) so introduced can be removed later if necessary (from the charcoal extracts) with much less labour than is involved in purifying the charcoal, and there has seemed to be no substantial loss of activity for vitamin B_1, in the 400 kg. tried. The fate of the vitamin B_4 has not yet been tested.

[1] The volume suitable for our receptacles.

[2] See note p. 230.

Elution of vitamin from charcoals.

"Acid" charcoal (vitamin B_4). The acid alcohol containing the charcoal is heated on the water-bath for 30 minutes, the temperature gradually rising to 70°. The mixture is then filtered through a Büchner funnel. The charcoal is re-extracted 3 times with acid alcohol. The filtrate is transferred to a 2 litre distilling flask and alcohol removed *in vacuo* at 45°. (The alcohol in the distillate is 75 % approximately and can be used for subsequent extractions.) To the final volume of 3 litres, NaOH is added to p_H 3·0 and the solution kept in cold store. The extract should contain about 18,000 day doses of vitamin B_4 together with 3000 day doses of vitamin B_1.

"p_H 7·0" charcoal (vitamin B_1). The 1200 g. charcoal, obtained at p_H 7·0, are extracted with $N/2$ HCl in four 1500 cc. beakers. We have found it best to use the steam-heated boiler as a water-bath for this purpose and to extract at 80° for 10–15 minutes, with constant stirring. The contents are sucked off on Büchner funnels; 3 suffice for 1200 g. Two further extracts are made with 2000 cc. of $N/10$ HCl. The combined filtrates (volume 6 litres approx.) are brought to p_H 3·0 ± 0·2 (bromophenol blue) and concentrated to 1500 cc. over a naked flame or on the water-bath in basins. 10 % barium chloride should be added before and during the process of concentration to remove sulphate as it appears. The extract contains 20,000–24,000 pigeon day doses of vitamin B_1, together with some vitamin B_4. It is only faintly coloured and may be concentrated to a convenient volume. Neutralisation to p_H 5·5 sometimes causes the formation of a rather large precipitate, consisting in part of Mg compounds. The amount varies and seems to depend especially upon the length of time during which the extract stands at p_H 1·0 before charcoal adsorption. The precipitate can be readily removed by centrifuge and washed without loss of activity. After this, the extract may be either (1) used directly to prepare crystals of high vitamin B_1 activity [Kinnersley, O'Brien and Peters, 1932], or (2) stored after addition of acid to p_H 3·5 and alcohol to 75 %, under which conditions it keeps well.

Before administration to animals (or patients) it is advisable to test for metals with H_2S at p_H 4·0. If present, they should be removed by treatment with H_2S at p_H 4·0 and filtration. The extract can be reacidified to p_H 3·0 with HCl and the H_2S removed either by passing nitrogen or by boiling. Removal *in vacuo* is apt to lead to troublesome frothing. The theoretical objection to boiling with H_2S present is of no consequence in this connection, as we have repeatedly found no loss of activity by boiling such solutions for an hour or more at p_H 3·0–4·0.

The $N/10$ HCl extract is the purer source of vitamin B_1 at this stage; 100 pigeon doses contain 25–50 mg. organic solids. It cannot be dried, but solids can be estimated by drying at 105° at which temperature the vitamin is not volatile.

A further supply of more impure vitamin B_1 is obtained together with vitamin B_4 by a further extraction with 50 % acid alcohol.

Extraction of p_H 7·0 "charcoal" by 50 % acid alcohol. The charcoals are extracted 4 times with 1400 cc. 50 % (by vol.) alcohol containing $N/10$ HCl, the extractions being made, as before, for 15 minutes at 80°. The combined filtrates are concentrated on the water-bath to avoid the frothing usually experienced *in vacuo*. The p_H is brought to 3·0, and removal of metals carried out if necessary as previously advised for $N/10$ HCl extracts.

This extract contains 10,000–12,000 doses vitamin B_1 together with vitamin B_4. It is very useful as a cruder source of vitamin B_1, either for administration to patients, or as a basal source of vitamin B_1 in rat and pigeon experiments. In the latter cases, the vitamin B_4 (and B_5), *etc.* present are often of definite value.

Summary of the labour required.

The extraction of 2 cwt. (100 kg.) yeast takes the time of one worker for one day. The subsequent processes from lead acetate to charcoal adsorption, two workers for one day; the extraction of the charcoal one worker for ½ day. It is an advantage to run continuously for 3 days (300 kg.) in all. In this case the extraction of the 2nd and 3rd 100 kg. on the second and third days needs one worker, making 3 in all for 3 days.

Summary.

Stage	100 kg. yeast: Volume litres	No. of workers	Days
A. Aqueous extraction of yeast	124–7	1	1
B. Lead precipitation and removal	124	.	.
C. Baryta clearance	120	.	.
D. $BaSO_4$	120–2	2	1
E. Acid charcoal	120–2	.	.
F. p_H 7·0 charcoal	122	.	.
G. Extraction of charcoals	.	1	½

Discussion.

1. The process outlined here, has been tested upon about 2500 kg. of yeast. Using the standard baker's yeast, we have never had a failure. Some workers use brewer's yeast for this preparation. In a modification for instance of our process by Chick and Roscoe [1929], adopted recently by Heyroth [1932], brewer's yeast is washed, and the extract is freed from metals before adsorption upon charcoal by treatment with H_2S. Theoretically perhaps this sounds an ideal procedure; in practice, we believe that the gain is illusory, at the expense of the very considerable labour. By this modification, 120 litres of solution require treatment with H_2S, instead of some 3–4 litres, which is the volume after adsorption. In the preparation of crystalline vitamin B_1, the H_2S stage may often be entirely eliminated by the use of sodium phosphotungstate [see Kinnersley, O'Brien and Peters, 1932].

2. The method of preparation here given leads to a vitamin B_1 concentrate still contaminated with vitamin B_4. If freedom from this is desired, the mercury stage (previously described) [Kinnersley and Peters, 1927], must be substituted instead of the acid charcoal. The resulting liquid filters quite well through the battery of funnels. Mercury present after adsorption is removed by treatment with H_2S. The mercuric sulphate precipitate can be used for the preparation of vitamin B_4 concentrates [*cf.* Reader, 1930].

3. For the preparation of concentrates of vitamin $B_1 + B_4$ for rat work upon vitamin B_2, *etc.*, it is best to use the original Kinnersley and Peters shortened process. The "acid" charcoal adsorption is performed at p_H 2·5 instead of p_H 1·0. Subsequent elution is made with acid 50 % alcohol (until acid to Congo red). The remainder of the procedure is as in the text for 50 % alcohol concentrates. Some 40,000 pigeon doses of B_1 may be obtained in this way[1].

This concentrate can also be used for clinical purposes.

4. Adsorption upon fuller's earth (Seidell) has been used, following a lead acetate precipitation [*cf.* Guha, 1931]. In our hands, however, there was no gain in time as compared with the charcoal adsorption technique.

[1] Probably use could be made of the lead acetate precipitate for the preparation of glutathione or vitamin B_2 [see Narayanan and Drummond, 1930; Chick and Roscoe, 1929].

SUMMARY.

The complete details are given for obtaining rapidly concentrations upon a large scale of vitamin B_1 and B_4 by the charcoal adsorption method from baker's yeast. Modifications suitable for various phases of work are also described.

We are indebted to the D.C.L. company for facilities in the supply of yeast, to the Medical Research Council for personal grants (V. R. and H. W. K.), and to the latter, the Christopher Welch Trustees, the Government Grant Committee of the Royal Society and the Department of Scientific and Industrial Research for grants for expenses. We are also grateful to many in the laboratory for help, especially to the skilful assistance of J. T. Cox, W. T. Turner, and R. W. Wakelin, also to Dr E. Walker for advice. Without their willing co-operation, the work could not have been brought to a successful conclusion.

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XXXV. CRYSTALLINE PREPARATIONS OF VITAMIN B_1 FROM BAKER'S YEAST.

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Recently [1932, 1, 2] we have referred to the preparation of crystals of high vitamin B_1 activity, which by comparative tests are more potent than those of Jansen and Donath [1926] and of Windaus *et al.* [1931; 1932]. The work of the latter authors, together with that of Ohdake [1931; 1932] appeared during the progress of our work [see also Van Ween, 1931; 1932, 1, 2]. In our belief, the claim that vitamin B_1 itself has been prepared requires re-examination. We here describe our methods of obtaining these crystalline plates from "active" charcoals. The average activity in terms of day doses for our pigeons by the mouth[1] has varied from 1·6 to 2·8 γ in the most recent experiments, 1·6 γ representing the greatest activity reached.

In all, we have a yield of some 500 mg. of crystals from 2000 kg. of baker's yeast. A special feature of our method is the use of sodium phosphotungstate at two stages and of alcohol at one to concentrate from an activity of approximately 0·3 mg. to 0·01 mg. No benzoylation is needed, and H_2S can be avoided if desired down to an activity of 0·005–0·01 mg.

Use is made in the separation of the following properties of vitamin B_1 alone; precipitation by sodium phosphotungstate at p_H 4–5·5, solubility in alcohol at p_H 3·0 and insolubility of the gold salt in aqueous solution at p_H 2·5. The description applies to aqueous HCl extracts of the "active" charcoals. The cruder 50 % alcohol extracts are not so suitable and have not been much studied.

Preparation.

All volumes recorded are approximate and are apt to vary slightly in different preparations.

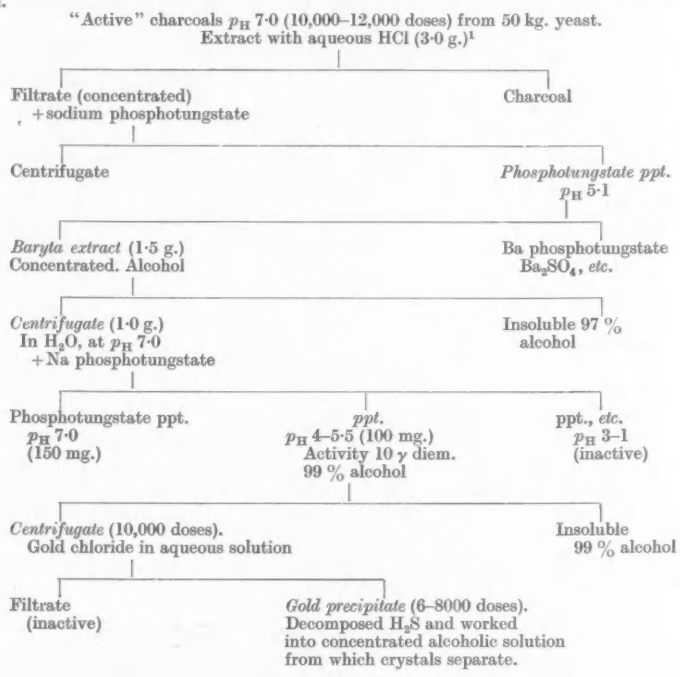
The stages in preparation of "active" charcoals are fully described in an accompanying paper [Kinnersley, O'Brien, Peters and Reader, 1933]. The "p_H 7·0" charcoals containing vitamin B_1 are extracted with $N/2$ HCl and $N/10$ HCl in succession. The combined and nearly colourless filtrates from 50·5 kg. (1 cwt.) of baker's yeast have a volume of about 1500 cc. After adding NaOH to p_H 2·0, they are concentrated on the water-bath or with naked flame to 750 cc., and cooled.

20 % NaOH is added cautiously to bring to p_H 5·0. Any precipitate which appears during this process is removed by centrifuge and washed with water. (The washings sometimes require addition of sodium sulphate to flocculate for filtration or centrifuging.) To the combined centrifugate and washings, a clear fluid of volume 750–1000 cc., are added 250 cc. of 10 % Merck's best phospho-

[1] One day dose is about one international vitamin B_1 unit.

Plan of preparation.

The reagents required are alcohol, phosphotungstic acid, gold chloride, baryta, and acid and alkali.



tungstic acid (previously brought to p_H 5·0 with NaOH)[2]. The phosphotungstate is added in slight excess and then 20 % H_2SO_4 and phosphotungstate until p_H 1–2·0 is reached. The precipitate so formed is allowed to stand overnight and settles to the bottom of the beaker. The supernatant fluid is often coloured. This has no effect upon the activity; practically all the vitamin B_1 present in the aqueous HCl extract (some 10,000–12,000 day doses) passes into the precipitate.

It is to be noted that the phosphotungstates thus precipitated are only a small proportion of the total bases precipitable by phosphotungstic acid in 5 % H_2SO_4, as will be readily seen by adding this reagent to the filtrate from the above.

The phosphotungstate precipitate p_H 1–5 is collected in two or four 100 cc. centrifuge-tubes, according to the quantity of precipitate. An amount of powdered baryta rather larger in volume than the precipitate is added to each tube, and after well grinding with a large glass rod in the shape of a pestle, the grinding is continued with the addition of 25 cc. H_2O, the mass centrifuged and the supernatant fluid filtered; the filtrate is kept acid to Congo red by additions of sulphuric acid during the filtration. The grindings with water are continued until the extracts are almost colourless (4–6 as a rule). Addition of H_2SO_4 is so arranged

[1] Organic solids. [2] 10 g. phosphotungstic acid need approximately 4·5 cc. 5*N* NaOH.

that the final p_H is just acid to Congo red. The combined filtrates (250 cc.) are then heated on the water-bath and centrifuged, the $BaSO_4$ being washed with $N/1000$ H_2SO_4.

One author [Guha, 1931] has criticised our use of solid baryta (rather than acetone) at this stage. In our opinion, the older technique amply repays the extra labour by the consistency and excellence of the yields [see Kinnersley and Peters, 1928].

Alcohol. The extract (250 cc.) is concentrated on the water-bath at p_H 3·0 with addition of 25 cc. 10 % $BaCl_2$ drop by drop to very slight excess, finally *in vacuo* to 1–2 cc. 100 cc. absolute alcohol are then added, and the whole is allowed to cool thoroughly at 0°; the inactive insoluble precipitate is then separated by the centrifuge.

Second phosphotungstate precipitation. Alcohol is removed *in vacuo* after addition of 10 cc. water, and the volume brought to 100 cc. with more H_2O. $5N$ NaOH is added to p_H 7·0[1] and then 10 cc. of pure 10 % sodium 1 : 24-phosphotungstate [see Barnes and Peters, 1932].

The p_H 7·0 precipitate is allowed to settle for not more than 30 minutes, and extracted with $Ba(OH)_2$ as above. The filtrate from this p_H 7·0 precipitate is reprecipitated by bringing to p_H 7·0 with NaOH and adding 10 cc. Na phosphotungstate as before. The combined filtrates from the two p_H 7·0 precipitates, which contain 95 % or more of the vitamin B_1 present, volume 200–250 cc., are treated gradually with approximately 20 cc. $N/10$ H_2SO_4 from a burette until the p_H is about 4·0. Fresh precipitation of phosphotungstate commences at approximately p_H 5·5[2], after addition of 10 cc. H_2SO_4. More phosphotungstate is added, if necessary to maximum precipitation.

The precipitate at p_H 5·5–4·0 is allowed to settle for several hours (best overnight) and contains 95 % at least of the vitamin B_1 in the original HCl extract. Generally no loss is detectable. It is extracted by grinding with baryta, and freed from $BaSO_4$ as before; the filtrate is adjusted to p_H 3·0 with $N/10$ $Ba(OH)_2$ and is concentrated upon the water-bath with addition of $BaCl_2$ in slight excess; finally it is taken to dryness *in vacuo* and extracted with 100 cc. absolute alcohol. After standing 7 hours at 0°, the slight insoluble and inactive precipitate is removed by centrifuging.

Gold precipitation. Alcohol is removed *in vacuo* at 40° and water added to 15 cc., any insoluble matter being removed by centrifuge. At p_H 3·0 ± 0·2, 2·5 cc. 10 % aqueous gold chloride are added, and the whole is allowed to stand for 1 hour in the ice-chest. It is then centrifuged and decanted. The centrifugate can be discarded. The gold precipitate is suspended in 15 cc. $N/200$ HCl and treated with H_2S for 1–2 hours. The sulphide is removed by a small filter, and nitrogen passed to remove H_2S. About 5 cc. $N/10$ $Ba(OH)_2$ are then added to p_H 3·5, followed by one drop 10 % $BaCl_2$ and the whole is concentrated on the water-bath to 0·3–0·5 cc. 5 cc. absolute alcohol are then added, the mixture warmed and washed quantitatively into a small 7–8 cc. test-tube, cooled, and centrifuged. The supernatant fluid is decanted, and the insoluble substances heated with 3 drops H_2O and 2 cc. absolute alcohol and cooled.

For maintenance of full activity, it is important to keep barium chloride in slight excess to minimise chances of excess of H_2SO_4, which is very dangerous to the activity. After this stage, the vitamin itself becomes somewhat insoluble in absolute alcohol [*cf.* Van Ween, 1932, 1, 2]. Barium chloride can be separated because of its greater insolubility. It is impossible to crystallise without

[1] Important not to make more alkaline.

[2] p_H 5 judged by plate, using the smallest possible drops.

the use of gold chloride. It will be noted that the description for the gold stage is quite different from that of Windaus *et al.* [1932]. Our preparation must evidently be purer before this treatment is started. The gold salts can be recrystallised, but it is safer to reserve such treatments for the hydrochloride. It seems impossible to avoid some loss of activity at the gold stage; the reason for this is not yet clear. We always employ glass-distilled water and redistilled alcohol.

Preparation of crystals. The combined extracts containing 70–80 % of the activity are added in portions of about 1 cc. at a time to another tube and concentrated after each addition until the final volume is about 2 cc. By this means and by addition of more absolute alcohol one or more small inactive precipitates may be obtained. The bulk of the water is removed in this way, but since there is a very small margin between removal of the last trace of $BaCl_2$ and appearance of active crystals this step requires practice. After removal of the last trace of $BaCl_2$, the crystals should appear as flat plates from a warm 98 % alcohol solution in half an hour, but it is advisable to leave in the ice-chest overnight. Decant, wash and centrifuge. 15–20 mg. of crystals (hydrochloride) are obtained of activity about $2{\cdot}3\gamma$ containing 6–8000 day doses of vitamin B_1. They can be recrystallised by adding one drop of H_2O, in which 15–20 mg. crystals will dissolve and making up to 2 cc. with absolute alcohol made acid with conc. HCl to p_H 3·0 (theoretical). The object of the acid is to inhibit hydrolysis and inactivation of the vitamin. When dried, the plates are apt to shrivel.

From more dilute solutions in absolute alcohol, crystallisation can often be induced by a layer of light petroleum; ethyl ether will not do. In this case, needles appear, which become plates upon recrystallisation.

The above technique has been applied to a large number of 50 kg. batches with uniform success and also to aqueous HCl extracts of "active" charcoals worked up by the older technique involving a mercuric sulphate stage, instead of the acid charcoal. The yield in this case has varied from 15 to 17 mg. per 50 kg. It is of course both possible and advantageous to work batches of 100–200 kg. through certain of these stages.

Stability and test solutions.

So far as is known, the crystals retain their stability in a desiccator over calcium chloride at room temperature for 2–3 months.

For purposes of test, solutions should be made up in absolute alcohol containing HCl (0·1 cc. of conc. HCl in 100 cc.), or in $N/1000$ HCl containing 20 % alcohol by volume for preservation. Such solutions keep well in cold store. Solutions tested at stages in fractionation when excess of barium is present, must be treated with H_2SO_4 in suitable strength to remove barium before dosing.

Estimation of activity.

The curative pigeon test has been used throughout, doses being given by mouth and reckoned as day doses for cure and protection. Occasional tests by injection indicate that a given sample of vitamin B_1 is about 30 % more active by injection than by mouth. In favour of the oral route, we have the better approach to physiological normality and the lessened risk of liberating tissue vitamin by trauma. For soluble preparations the standard deviation (σ) is 44 as a maximum, reckoning the mean as 100. In a recent test upon 10 birds for instance, the average day dose was 5·4 and the standard deviation[1] (σ) 2·36. From this can be calculated the standard error of the mean[2] (ϵ) with different

[1] $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ d = difference from mean, n = number of observations.

[2] $\epsilon = \frac{\sigma}{\sqrt{n}}$.

numbers of animals upon test; in the Tables are given the values of the mean and of 2ϵ; 21/22 of such tests should fall within the range $\pm 2\epsilon$. This is probably a severe estimate of the probable accuracy. We have noticed that some preparations give results with much smaller standard deviation, but have not enough evidence to decide whether this is significant. With solids as source of vitamin B_1, larger variations are often found, probably related to the removal of vitamin from the solid in the gut. In the latter case the relation between time of cure and dose is much obscured. This has led to a revision of the figure given for the day dose [Peters *et al.*, 1931] in terms of the International B_1 Unit. In tests not yet complete, a revised figure indicates one pigeon day dose (under our conditions) to be approximately equivalent to 1 International Unit (instead of 1·5). Pending a settlement, we have adhered here to the day dose unit.

It will be noticed that many of the tests in Table I give an average cure of 5–6 days, and are therefore tested at points of equal biological effect.

Table I. *Vitamin B_1 potency of crystals (hydrochloride).*

Prep. no.	Melting-point (4)	Dose γ	Average day cure	No. of birds	Day dose γ	2ϵ (1)	Remarks
25.11	230°	17·0	6·5	4	2·6	±1·14	"Acid" charcoal method
28.4		12·0	5·2	6	2·3	±0·78	" "
53.A.4		13·8	5·5	10	2·5	±0·70	" "
56.20		14·0	5·0	5	2·8	±1·09	" "
57.7	222°	11·5	5·0	4	2·3	±1·01	" "
43.17		10·0	6·8	6	1·5	±0·51	" "
63.A.9	221–3°	14·0	6·1	4	2·3	±1·01	Mercuric sulphate instead of "acid" charcoal
64.19	225–30°	14·6	6·6	4	2·2	±0·97	Mercuric sulphate instead of "acid" charcoal
42.A	221–50°	9·0	4·5	7	2·0	±0·66	Refractionated with Na phosphotungstate
61.A.15	221°	9·0	5·6	9	1·6	±0·46	Refractionated with Na phosphotungstate
G. (2) (Windaus *et al.*)		14·2	3·3	10	4·31	±1·21	For the 18 tests
		23–28	5·6	8	4·58	±1·42	=4·45 ±0·93.
J. (3) (Jansen)		22·0	3·7	4	6·0	±2·8	

(1) 21/22 tests would fall within 2ϵ.
(2) Preparation kindly supplied by Dr Tschesche (method of Windaus *et al.*).
(3) Preparation kindly supplied by Dr Jansen (method of Jansen and Donath).
(4) M.P. quite sharp in case of 57.7, 61.A.15, 63.A.9. Remainder indefinite.

Comparison of activities.

Table I gives the estimated potency of various samples of crystals. An average of all our tests, which were oral, gives $2{\cdot}17\gamma \pm 0{\cdot}11$. It is certainly a striking circumstance that our values should be so near those for tests "by injection" given by Windaus *et al.* [1932] as $2{\cdot}4\gamma$, and by Ohdake [1932] as of "order of $2{\cdot}5\gamma$." Nevertheless we consider this to be coincidence. Firstly, oral tests usually give a lower response than tests by injection. Our results by injection would give an average under 2γ. Secondly, direct comparison by simultaneous tests upon our pigeons indicated a much higher potency for 53.A.4 than for G. As calculated above, we have assumed a maximum standard deviation of 44 % for G; actually these tests gave a smaller standard deviation by direct calculation. The difference between 53.A.4 and G is significant. A few tests upon

a new preparation made by Jansen gave a higher potency than previously [Jansen *et al.*, 1930], but slightly lower than G. Thirdly, in more than one case we have ourselves reached crystals of greater potency than 2γ. 61.A.15 for instance gives an average of $1{\cdot}6\gamma \pm 0{\cdot}46$, the difference between this value and that of 53.A.4 is again significant. Hence, even if we decided to set aside the cogent evidence from direct comparison, we still have to account for the greater activity of 61.A.15. This leads to the conclusion that crystals more potent than the alleged vitamin B_1 can be obtained by the charcoal-sodium phosphotungstate technique.

Properties of crystals.

Precipitation and colour reactions. Table II shows that these follow those described by others. It is interesting to note that, in confirmation of earlier assertions by two of us, there is now general agreement that the present vitamin B_1 preparations do not give precipitates with mercuric sulphate in acid solution. In Table II the limits for the reaction have been added, where these are known.

Table II.

Precipitating substance	Van Ween	Ohdake	Windaus *et al.*	Present crystals	Remarks
Phosphotungstic acid	1/10,000	+	+	+	
Na phosphotungstate p_H 5·0	.	.	.	+	1/100,000–1/1,000,000
$AgNO_3 + Ba(OH)_2$	.	+	+	.	
$HgCl_2$ (aqueous)	.	+	+	+	In presence of Na acetate 1/10,000. Redissolves immediately in absence
$HgSO_4$ (acid)	−	−	−	−	
Picric acid (aqueous)	.	−	−	−	
Picrolonic acid	.	+	+	+	
Gold chloride (aqueous)	1/10,000	+	+	+	
Platinic chloride (alcoholic)	+	+	+	+	
Flavianic acid	.	−	−	−	
Rufianic acid	.	.	+	.	
Iodine KI	.	+	.	±	Rather indefinite ppt.
Dragendorff	.	+	.	+	
Colour reactions.					
Pauly (diazo)	Yellow-brown	+[1]	Nil	Yellow	Technique of Koessler and Hanke [1919]. 10γ used.
Hunter (ergothioneine)	.	.	.	−	22γ used
Ninhydrin	.	−	.	−	In p_H 7·0 phosphate buffer; 20γ alanine good reaction; 44γ vitamin B_1 yellowish-green only. 0·05 cc. 0·1 % ninhydrin, 0·6 cc. solution
Bial (pentose)	.	.	.	−	132γ in 0·1 cc. +0·2 cc. Bial's reagent. 10γ arabinose good reaction under same conditions
Sakaguchi (arginine)	.	−	−	±	44γ in 0·1 cc. +1·0 cc. *N*/10 NaOH +0·05 cc. 0·15 % naphthol, 0·05 cc. 2 % hypochlorite; faint reaction less than $2{\cdot}5\gamma$ arginine nitrate
Sulphur–SH (direct)	.	−	−	−	
Sulphur–SH (after heating with alkali)	.	+	+	+	10γ used

[1] Differed from histidine.

Sulphur reactions. The nitroprusside S reaction is given by addition of ammonium sulphate, ammonia and sodium nitroprusside after some 20 minutes' heating on the water-bath with 20 % alkali, or after fusion with sodium, but not well by rapid boiling with NaOH. In this, it differs from cystine. Some preparations showed the reaction much less intensely than others. Preliminary heating with ferric chloride did not restore the Pauly reaction (difference from ergothioneine). It may be noted that by reduction of scale, 0·1 cc. of solution *plus* one drop of 40 % NaOH, the nitroprusside reaction can be detected in 10γ

or less of vitamin B_1 crystals and about 2γ of cystine. After heating and cooling, a few crystals of ammonium sulphate are added and then dilute nitroprusside.

Sakaguchi reaction. Some preparations gave negative results; where present it is probably due to traces of impurity carried over from earlier stages, which give the reaction intensely.

Properties of crystals. When precipitated from dilute alcoholic solutions with light petroleum, the crystals are needle-shaped. From acid alcohol however, by the technique here given, the crystals always form beautiful plates, strikingly like the photographs of Ohdake [1932], and give an impression of purity. With the exception of Ohdake's Pauly reaction, their behaviour to precipitants and general reactions appears similar to those of others. Yet we have evidence that they are not pure. Their biological potency has been shown to vary. The melting-point of the recrystallised dihydrochloride never reaches 250°, that given by others. We have obtained crystals with quite sharp melting-points of 221°. The M.P. of recrystallised picrolonates has varied, values of 165°, 221° and 229° having been observed. In face of the greater activity, this cannot mean that we merely have impure vitamin B_1. It might be due to slight differences in hydrochloride composition, as in the well-known case of histidine; but there is other evidence of difference. Preparation G is stated to have an absorption band at 260 $m\mu$ [Windaus *et al.*, 1932]. In unpublished work in 1927 (upon impure specimens) Dr C. W. Carter in this laboratory found absorption at 247·5 $m\mu$ which did not change upon heating with alkali, although all vitamin B_1 activity had disappeared. Recently Mr Philpot with our crystalline preparations has found maximum absorption at 248/9 $m\mu$. Therefore the absorption spectrum of our crystals is different from that described by others [*cf.* Heyroth and Loufbarrow, 1932; Guha, 1931].

Further, preliminary experiments show that some crystals of vitamin B_1 are still contaminated with vitamin B_4[1] which is stated to contain no S [*cf.* Barnes, O'Brien and Reader, 1932]. Hence vitamin B_1 itself must be part only of our preparations, *i.e.* potent in doses of 1γ or under. This leads again to the unfortunate conclusion that neither ourselves nor others have yet reached pure vitamin B_1. Elaborate analyses are of little value, until consistency of biological and chemical composition has been proved. For comparison we quote without comment and with no emphasis the following analyses by Dr Ing Schoeller made upon various samples of crystals dried *in vacuo* at 80°. Weight lost at 80° approximately 3·0 %. There was a residue of $1{\cdot}0 \pm 0{\cdot}5$ % (compare Van Ween [1932, 2, p. 127] as to difficulty of freeing from traces of residue).

Table III.

	C	H	N	S	Cl
Present crystals	42·6	5·53	14·5	9·45	20·92
	41·8	5·82	15·2	9·36	—
	—	—	15·1	9·81	—
Average	42·2	5·7	14·9	9·5	—
Ohdake (Average)	40·87	5·53	15·94	9·26	20·3
Van Ween (Average)	40·31	6·32	15·71	8·71	19·77
Windaus *et al.*	40·52	5·68	14·96	8·92	19·78

It will be seen that there is a general agreement, but variations from other figures large enough to be of consequence. There is a distinct tendency for higher carbon and sulphur values.

[1] Personal communication by Mrs Walker.

By several lines of evidence, the conclusion is reached that the crystals made by the methods described in this paper are more potent than and different from those of others. Three possibilities remain, and are now receiving attention.

(1) All crystals hitherto made are a mixture of active and inactive vitamin B_1. This is hard to reconcile with the conflicting position of the ultra-violet bands, presuming these to represent part of the vitamin molecule.

(2) The crystals contain vitamin B_1 as impurity. In this case the activity of the true vitamin could be at least $0{\cdot}1\gamma$ in our terms.

(3) More than one compound can function as vitamin B_1. In this event, we might have either a base able to exist free or in combination with other substance, or different bases with identical biological functions.

SUMMARY.

Methods of making crystalline preparations of high vitamin B_1 activity are described in detail, based upon the principle of separation by sodium phosphotungstate at p_H 4·0–5·0 followed by gold chloride and crystallisation from alcohol. Comparatively few stages are needed to concentrate from the activated charcoal stage. Approximately 500 mg. have been made from 2000 kg. baker's yeast. The crystals vary in activity, the highest potency being $1{\cdot}6\gamma \pm 0{\cdot}4$ for a day dose, orally administered to pigeons. This is greater than the activity of other crystalline preparations previously described.

We are grateful to the Medical Research Council for personal grants to one of us, H. W. K., and for grants for expenses to the Government Grant Committee of the Royal Society, the Department of Scientific and Industrial Research and the Christopher Welch Trustees. We also thank T. F. Clarke, J. T. Cox, W. T. Turner and R. W. Wakelin for skilful assistance.

We are also indebted to the D.C.L. Company for facilities granted in the supply of baker's yeast.

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XXXVI. THE DESIGN OF EXPERIMENTAL PERCOLATING FILTERS.

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(*Received January 5th, 1933.*)

In the course of an investigation of methods of purification of effluents from beet sugar factories, which has been in progress at the Rothamsted Experimental Station during the past five years, the oxidation of solutions of carbohydrates by the process of biological filtration on percolating filters has been systematically studied. The different kinds of small laboratory percolating filters constructed specially for the experiments may be of value to other investigators in similar experimental work on the purification of sewage and various industrial waste waters. An account is therefore given in the following pages of the different filters and the circumstances in which they might be advantageously employed.

Drainpipes of earthenware or cement are obtainable in a variety of sizes. They have been found useful as containers for filtering medium in preliminary work when comparisons were desired of the effect of changing the grade of size or type of medium or the nature of the solution to be filtered. Dawson, in experiments at Rothamsted with aqueous extracts of sugar beet and of mangolds (not published) used a filter in which the medium was held in a cylinder of iron wire gauze; samples of liquid at different levels of the filter were withdrawn through small glass funnels inserted through the gauze. Dawson's experiments showed that most of the carbohydrate in solution was oxidised in the upper half of the filter. Chick [1905] made use of filters built up from glass cylindrical sections in order to study the process of nitrification in sewage. Glass funnels were arranged at various depths of the filters so that samples could be taken of the solutions undergoing filtration. These experiments led Chick to conclude that the process of nitrification is a rapid biological action which takes place in two stages, *viz.* oxidation of ammonia to nitrite and oxidation of nitrite to nitrate.

Filters built in sections separated by air spaces. It is sometimes of interest to study the progress of purification of a solution during the process of biological filtration, and for this purpose filters built in sections possess the advantage that samples of the liquid leaving each section can readily be obtained. The division of a percolating filter into a number of separate sections appears to have been introduced in 1898 by Scott-Moncrieff [1909] in order to produce a well-oxidised effluent from the strong sewage of the Caterham Barracks. This investigator used a large scale installation of four filters, each 11 ft. by 9·5 ft. and 5 ft. deep. The clinker filtering medium was arranged on seven perforated trays separated from one another by air spaces. After the sewage had passed through two septic tanks the liquor was treated in the sectional filter at a rate of 31 g.y.d.[1]

[1] Gallons of liquid filtered per cubic yard of filtering material per day.

and a well nitrated effluent was obtained. In general it was found that the upper half of the complete filter removed more organic matter than the lower half, while the latter produced the greater quantity of oxidised nitrogen. Barritt [1931], who first made use of separate sections of earthenware pipes in order to study the rate of oxidation of sucrose solutions, found that most of the carbohydrate was oxidised in the upper half of the filter. Similar results were reported by Jenkins [1931] in a study of the oxidation in sectional percolating filters of solutions containing sucrose and ammonium chloride.

For certain purposes the method of splitting up a single filter into separate sections divided by air spaces suffers from the disadvantage that the results are not strictly comparable with those obtained with non-sectional filters for the following reasons: (1) separation of the sections does not permit the upward movement of organisms which is possible in the non-sectional type of filter, (2) the air spaces between the sections may affect the amount of air passing through the filters and may alter the distribution of liquid in the filtering medium. For example, the sectional filter previously used by the author [1931] consisted of six separate cylindrical glass parts, each 6″ in diameter and 9″ deep, with a funnel-shaped bottom supporting the medium. The liquid, which dropped on to a section, collected in the funnel and fell on to the centre of the surface of the next lower section, and then spread through the medium. Uniform distribution of the liquid over the top surface of each section was not, therefore, obtained. Thus, the percentage purifications of 0·1 % solutions of sucrose filtered through approximately the same depth of medium and at the same rate of flow in (*a*) a non-sectional filter, (*b*) three sections of a sectional filter, were 95–100 % and 66 % respectively.

Sectional filter equivalent to a single filter. A new type of filter was accordingly designed and has been operated for 2 years. This filter is comparable with a non-sectional filter and yet samples of liquid at different depths may be collected by simple manipulation. The filter is illustrated in Fig. 1 and consists of six units of octagonal cross section. The sides of the octagon are of wood and of standard size and shape (Figs. 2 *a* and 2 *b*) and the top and bottom edges are bevelled at an angle of 60° to the horizontal. The eight sides of each unit are held together in position by two 1″ bands of iron, shrunk on and screwed down to each side (Fig. 2 *c*). The medium is supported in the section by means of a rustless steel tray made from Hadfield's C.R.I. steel. The tray, perforated and shaped as shown in Fig. 2 *d*, is placed inside the section and the flaps are screwed to the sides so that the tray is just flush with the bottom of the section. Each section is supported in a frame (Fig. 1) by two separate wooden pegs which are pushed through holes in the frame into sockets in blocks of wood screwed on to the sides of the octagon. Spare holes are made in the frame at suitable distances so that the sections can be lowered if necessary. The sections are filled with medium up to the level of the inside bevelling. When the sections are assembled, medium is in contact with both sides of the steel plates, and, if the holes in the plate are made large enough, particles of medium may project through from one section to the next.

Experiments on filtration are carried out with the apparatus in the position shown in Fig. 1. If samples are required from all sections, the final effluent is first sampled. The last section is then lowered and supported by the pegs in the spare holes and the effluent from the fifth section is then collected. A similar procedure is followed until the effluent from every section has been sampled.

Filters constructed entirely of glass. In experiments on the effect of adding phosphates to solutions of carbohydrate undergoing treatment in percolating

filters it is essential to exclude from the apparatus any material from which the liquid or organisms in the filter might extract phosphorus. Even resistant forms of phosphorus, such as are likely to be present in clinker and other media frequently employed, may become slowly available to bacteria and fungi. There

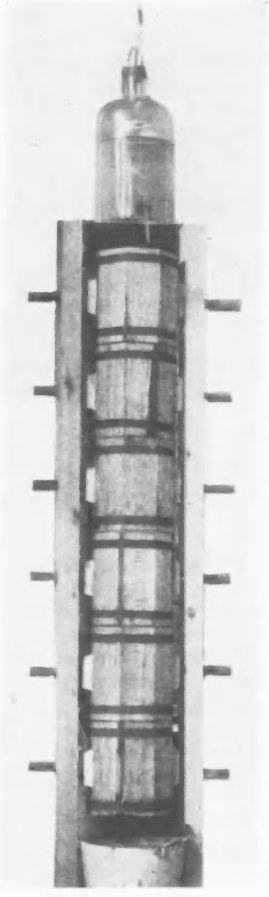

Fig. 1. Wooden percolating filter.

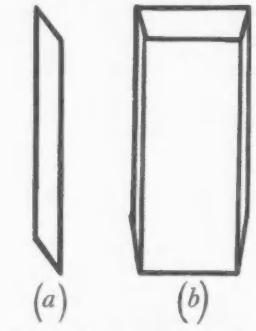

Fig. 2 *a*. End view of side of octagon; *b*. Front view of side of octagon.

Fig. 2 *c*.

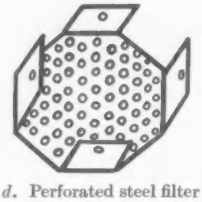

Fig. 2 *d*. Perforated steel filter plate.

are several non-phosphatic media obtainable, *e.g.* glass beads, which are not readily available in suitable sizes, and cullet or waste sheet glass, which is, however, difficult to break down and grade. On the suggestion of Mr E. H. Richards graded glass from broken bottles was tried in several experiments, since it is obtainable as an article of commerce. This medium was contained in

cylindrical lamp-glasses. A perforated aluminium plate was fastened to the bottom of each cylinder by means of a mixture of hot bitumen, pitch and a little tallow, and was secured in this position by a strip of aluminium which partly covered the bottom of the cylinder and partly overlapped the plate. The strip of aluminium was similar to the metallic covers used for fastening down the lids of preserved meat bottles. Any number of sections could be joined together by means of bands of rubber cut from motor car inner tubes. Each section was kept in position in a frame by a metal clip (Fig. 3 *a*). The last section rested in a glass funnel supported in a retort ring. This ring could be moved up and down

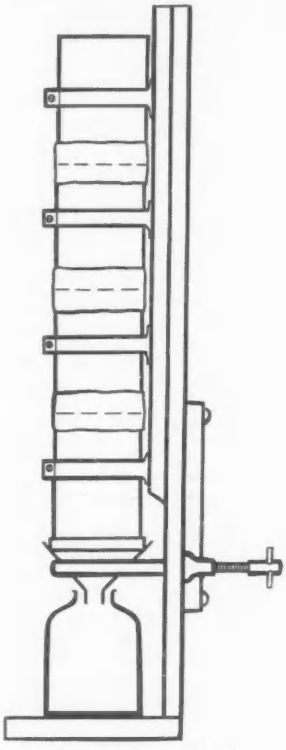

Fig. 3 *a*. Glass sectional filter.

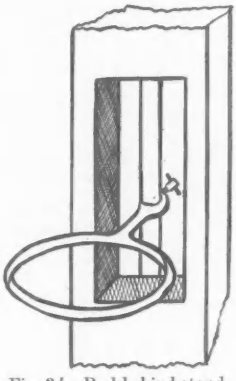

Fig. 3 *b*. Rod behind stand to support ring.

Fig. 3 *c*. Constant dripping apparatus.

a length of piping screwed on to the back of the stand (Fig. 3 *a* and *b*) so that the last section could be lowered and the effluents from any of the sections could be sampled.

The glass medium from broken bottles packed badly and filters which contained it choked after being supplied for only a short time with solutions of carbohydrate and nitrogenous compounds. At the suggestion of Dr A. Parker, the broken glass was replaced by short pieces of glass tubing. Tubes of 1 cm. bore with walls of 1 mm. thickness were cut into lengths varying from 1·5 to

2 cm. With this kind of medium the filters have proved very satisfactory. Owing to the large surface and air space there is very little tendency for the filters to choke, while dead film is not permitted to accumulate in the filter, but is rapidly washed out with the effluent.

In the various experiments, a modified form of Mariotte's bottle has been used (Fig. 3 *c*) for supplying liquid at a constant rate. The hydrostatic head in this apparatus is controllable by the frictional resistance offered to the passage of air through a length of thermometer capillary tubing. The four-way outlet permits of easy cleaning out of bacterial growths in the tubes.

Filters constructed entirely of glass have been used successfully for studies on the effect of filtering solutions of sucrose containing different amounts of phosphate, in order to determine the quantity of phosphorus required by a biological filter in decomposing a definite amount of sucrose. As glass medium does not interfere with the methods commonly employed for the analysis of nitrogen, phosphorus and potassium, it is possible to recover the whole of these elements contained in the film of a percolating filter. The results of experiments in which use has been made of filters constructed entirely of glass, will be published in due course.

It is a pleasure to acknowledge the assistance of Mr E. H. Richards and Mr D. M. T. Morland in the construction of the filters described in this paper. The apparatus was constructed for experiments carried out at Rothamsted Experimental Station as part of the programme of the Water Pollution Research Board, of the Department of Scientific and Industrial Research and the paper is published by permission of the Department.

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XXXVII. THE BIOLOGICAL OXIDATION OF CARBOHYDRATE SOLUTIONS.

II. THE OXIDATION OF SUCROSE IN THE PRESENCE OF DIFFERENT INORGANIC NITROGEN COMPOUNDS.

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(*Received January 5th, 1933.*)

THE important rôle of nitrogen in the oxidation of carbohydrate solutions by the method known as biological filtration, *i.e.* percolation of the solution through a bed of inert material, has been studied in detail at Rothamsted by Dawson, Jenkins and Martin [unpublished work]. In later work Barritt [1931, 1] concludes that a 0·2 % solution of sucrose is most effectively oxidised by a similar method provided the initial C:N ratio is not greater than 15/1. The former workers all used either ammonium salts or albumin as the source of nitrogen, and the solutions were made up in Harpenden tap-water. This water is derived from chalk measures and besides large quantities of calcium bicarbonate contains about 1 part of total nitrogen per 100,000, 0·5 part as nitrate and 0·5 part as organic nitrogen. In previous work it was quite evident that both forms of nitrogen were utilised by the organisms which develop on percolating filters, since nitrogen-free effluents were frequently obtained.

The objects of the experiments described in this paper were: (1) to study the effect of nitrogen supplied as ammonia, nitrite and nitrate on the biological oxidation of sucrose, (2) to ascertain the changes which these nitrogenous compounds undergo in a percolating filter and (3) to examine the effect of narrow C:N ratios with the carbohydrate and nitrogen compounds chosen.

Method of experiment.

The apparatus and its method of use have been described in the preceding paper [Jenkins, 1933]. The volume of the filter was 0·0342 cu. yd., so that a rate of 7 litres per day represented a flow of 46 gallons of solution per cubic yard of filter medium per day (abbreviated g.y.d.). The filter was filled with washed and weathered clinker of $\frac{1}{4}''$ to $\frac{1}{2}''$ grade.

Solutions of sugar and mineral salts were made up daily in distilled water and the desired quantity of the nitrogen compound was added. The amounts of sugar and salts were as follows:

	g. per litre
Sucrose	1·0000 (glucose equivalent 1·053)
$MgSO_4, 7H_2O$	0·0071
K_2HPO_4	0·0143
NaCl	0·0028
$CaCl_2, 6H_2O$	0·1375
Fe_2Cl_6	Trace

The filtration tests with sugar and the different compounds of nitrogen were made in two experiments. In the first experiment solutions of sugar *plus* sodium nitrite, followed by sugar *plus* sodium nitrate, and then by sugar *plus* ammonia, were filtered at a rate of 46 g.y.d. Each nitrogen compound was supplied in two concentrations, sufficient to provide C:N ratios of 8·4:1 and 4·2:1 respectively. In the second experiment the solutions were filtered at a rate of 186 g.y.d. and the order of filtration was sucrose *plus* ammonia (C:N ratio first 4·2:1, then 8·4:1), sucrose *plus* nitrate and sucrose *plus* nitrite. All the results presented in this paper were obtained with the same apparatus, so that comparisons may be made only between the effects of the nitrogen compounds at different stages of the history of the filter. The object of reversing in the second experiment the order in which the nitrogenous substances had been supplied in the first experiment was to eliminate the beneficial effect of improved oxidation with the ageing of the filter.

Methods of analysis.

Total sugars. A suitable quantity of sugar solution (1 to 20 cc.) was inverted in stout 6″ × 1″ test-tubes with 2 cc. *N* H_2SO_4 by heating for 20 to 25 minutes in a boiling water-bath. The solution was neutralised with 2 cc. *N* NaOH, made up to about 25 cc. with distilled water and 10 cc. Hagedorn-Jensen ferricyanide solution were added. The test-tubes were closed with rubber stoppers each with a small hole and were then heated for 5 mins. in a boiling water-bath, after which a rounded glass rod was inserted in the hole in the stopper and the heating continued for a further 5 mins. Experiment showed that the rubber stopper had no measurable effect on the results, while the cooled solution would keep without change for at least a day if the analysis could not be continued at once from this stage. The determination of the unreduced ferricyanide was made in the usual way except that the contents of the test-tubes were quickly transferred to small conical flasks just before titrating with *N*/100 thiosulphate. If (1) the minimum volume taken for analysis is 0·1 cc. and the maximum volume is 20 cc. and (2) titrations which differ from the thiosulphate figure for the blank determination on the ferricyanide by less than 0·4 cc. and from zero by less than 0·5 cc. are disregarded, then the concentrations of glucose which may be determined by this method range from 0·0004 to 1·25 %.

Invert sugar in the samples was anlaysed by the method described above with the omission of the inversion process.

Nitrogen as ammonia was estimated colorimetrically with Nessler solution when the concentration did not exceed 0·1 part N per 100,000. Above this figure the ammonia was determined by the distillation method.

Nitrite was determined colorimetrically with *meta*phenylenediamine.

Nitrate plus *nitrite*. The residue in the distillation flask after removal of ammonia was distilled with B.D.H. powdered Devarda alloy and magnesia into *N*/25 acid. It is advisable to carry out a blank determination on each batch of alloy.

Organic nitrogen was obtained by a Kjeldahl analysis on the residue left after the nitrite and nitrate had been distilled off as ammonia.

p_H was measured colorimetrically in the Hellige apparatus.

Exp. 1. Filtration at a rate of 46 g.y.d.

1st to 48th day: 5 parts N per 100,000 as $NaNO_2$, Series 1
49th to 90th day: 10 „ „ „ 2

91st to 100th day: 5 parts N per 100,000 as $NaNO_3$, Series 1
101st to 121st day: 10 „ „ „ 2
122nd to 147th day: 5 „ NH_4Cl, „ 1
148th to 177th day: 10 „ „ „ 2

Results of Exp. 1.

Sugar oxidation (*Table I A*). The filter did not appear to be mature after the 48th day of experiment at the end of Series 1, since it continued to improve with increased time of running. At a rate of 46 g.y.d. 97·9 % of the sugar was

Table I. *Composition of solution and of effluents from different sections of the filter: parts per 100,000 nutrient solution filtered: sucrose nominally equal to 105·3 parts glucose.*

Rate of filtration = 46 g.y.d.

A. Nitrogen added as sodium nitrite	Series 1 (1st to 48th day). Average results of 5 sets of analyses made on the 16th, 30th, 41st, 45th and 48th day. Amount of nitrogen added = 5 parts per 100,000. C:N ratio = 8·4:1							Series 2 (49th to 90th day). Average results of 3 sets of analyses made on the 56th, 63rd and 83rd day. Amount of nitrogen added = 10 parts per 100,000. C:N ratio = 4·2:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugars (calc. as glucose)	116·6	61·9	26·6	9·0	2·5	0·3	0·1	109·1	28·2	12·1	3·8	1·4	1·1	0·4
Sucrose (calc. as glucose)	116·6	29·7	1·8	3·7	0·9	0·1	0·0	109·1	1·8	1·2	0·8	0·3	0·6	0·2
Invert sugar (calc. as glucose)	0·0	32·2	24·8	5·3	1·6	0·2	0·1	0·0	26·4	10·9	3·0	1·1	0·5	0·2
Nitrogen as ammonia	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Nitrogen as nitrite	5·00	3·30	2·30	1·90	1·30	0·60	0·30	10·00	3·60	3·40	2·30	1·30	0·60	0·50
Nitrogen as nitrate	0·0	0·10	0·29	0·53	1·26	2·19	1·90	0·0	1·21	1·31	1·42	1·38	2·60	2·83
Total inorganic nitrogen	5·00	3·40	2·59	2·43	2·56	2·79	2·29	10·00	4·81	4·71	3·72	2·68	3·20	3·33
P_H	6·2	7·2	7·5	7·7	7·7	7·7	7·8	—	—	—	—	—	—	—

oxidised by the first four sections, whereas in the sectional filter previously used [Jenkins, 1931] only 86·3 % of a 0·1 % solution of sugar was oxidised at the same rate of feed when the filter was mature. In the early stages of the experiment a large percentage of the total sugar appeared as invert sugar. Thus the effluent from Section 1 contained 32·2 parts of invert sugar out of a total sugar content of 61·9 parts per 100,000, while the corresponding figures for the effluent from Section 2 were 24·8 and 26·6 respectively. Owing to the unexpected efficiency of the filter little sugar remained for oxidation in the lower sections.

Table I B. It would be erroneous to conclude from a comparison of the amounts of sugar left in the effluents from Section 1 in Table I A, Series 2, and Table I B, Series 1, that the change over from nitrite to nitrate had produced a beneficial effect. The film on the filter was still young at this stage and the individual analyses from which the average figures for Section 1, Table I A, Series 1 and 2, are obtained indicate that the amount of sugar which would have been oxidised with the continued use of nitrite as the source of nitrogen would have equalled that decomposed when nitrate was subsequently employed. With both proportions of nitrate-nitrogen supplied, nearly all the sugar was oxidised in the first section.

Table I (*cont.*)

B. Nitrogen added as sodium nitrate	Series 1 (91st to 100th day) Average results of 2 sets of analyses made on the 91st and 99th day Amount of nitrogen added = 5 parts per 100,000 C:N ratio = 8·4:1							Series 2 (101st to 121st day) Average results of 2 sets of analyses made on the 106th and 114th day Amount of nitrogen added = 10 parts per 100,000 C:N ratio = 4·2:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugars (calc. as glucose)	101·1	1·4	0·4	0·4	0·4	0·4	0·3	107·9	12·5	0·6	0·0	0·0	0·0	0·0
Sucrose (calc. as glucose)	101·1	1·2	0·1	0·2	0·2	0·3	0·2	107·9	6·1	0·5	0·0	0·0	0·0	0·0
Invert sugar (calc. as glucose)	0·0	0·2	0·3	0·2	0·2	0·1	0·1	0·0	6·4	0·1	0·0	0·0	0·0	0·0
Nitrogen as ammonia	0·0	0·03	0·04	0·04	0·04	0·02	0·02	0·0	0·01	0·01	0·02	0·01	0·00	0·01
Nitrogen as nitrite	0·03	0·17	0·16	0·08	0·03	0·01	0·01	0·05	2·30	1·56	1·10	0·65	0·14	0·01
Nitrogen as nitrate	4·97	1·32	0·68	0·93	0·84	1·38	1·60	9·95	3·39	3·15	3·26	3·34	3·24	3·12
Total inorganic nitrogen	5·00	1·52	0·88	1·05	0·91	1·41	1·63	10·00	5·70	4·72	4·38	4·00	3·38	3·14
P_H	6·2	7·2	7·3	7·5	7·5	7·6	7·6	7·2	7·3	7·4	7·6	7·6	7·6	7·6

Table I C. The amount of sugar oxidised in the filter was of the same order as that oxidised when nitrate was the source of nitrogen. Mr Yates of the Rothamsted Statistical Department, has examined the individual figures of analysis and has found that no significant difference exists between the results of oxidation of sugar in the presence of ammonia, nitrite or nitrate, at any level of the biological filter if the values found during the first 48 days are excluded from the examination. The differences between the results for sugar oxidised with any one form of nitrogen were found to be greater than the differences due to the use of various forms of nitrogen.

Nitrogen results, Table I A, B and C. At the beginning of the experiment, *Table I A, Series 1*, the complete removal of 1·6 parts of N in Section 1 from

Table I (*cont.*)

C. Nitrogen added as ammonium chloride	Series 1 (122nd to 147th day) Average results of 3 sets of analyses made on the 127th, 133rd and 140th day Amount of nitrogen added = 5 parts per 100,000 C:N ratio = 8·4:1							Series 2 (148th to 177th day) Average results of 3 sets of analyses made on the 148th, 155th and 177th day Amount of nitrogen added = 10 parts per 100,000 C:N ratio = 4·2:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugars (calc. as glucose)	102·2	11·5	3·1	0·6	0·1	0·1	0·0	105·4	10·8	4·2	2·3	1·1	0·6	0·4
Sucrose (calc. as glucose)	102·2	8·4	2·7	0·3	0·1	0·1	0·0	—	4·6	0·8	0·7	0·2	0·4	0·3
Invert sugar (calc. as glucose)	—	3·1	0·4	0·3	0·0	0·0	0·0	—	6·2	3·4	1·6	0·9	0·2	0·1
Nitrogen as ammonia	5·00	3·46	3·18	3·06	3·05	2·35	1·90	10·00	8·29	7·10	6·32	5·46	4·10	2·85
Nitrogen as nitrite	—	0·02	0·00	0·00	0·00	0·01	0·02	—	0·00	0·00	0·01	0·02	0·02	0·02
Nitrogen as nitrate	—	0·00	0·00	0·04	0·21	0·36	0·78	—	0·00	0·05	0·35	1·05	2·20	4·16
Total inorganic nitrogen	5·00	3·48	3·18	3·10	3·26	2·72	2·70	10·00	8·29	7·15	6·68	6·53	6·32	7·03
P_H	6·1	6·2	6·8	7·0	7·2	7·2	7·2	6·4	6·2	6·2	6·5	6·6	6·6	6·6

5 parts supplied as nitrite was probably caused by the direct assimilation of nitrogen by the growing micro-organisms. A smaller quantity of nitrogen was taken up or destroyed in Section 2, but beyond this part of the filter, where the concentration of carbohydrate was relatively small, the function of the filter was to oxidise nitrite to nitrate. There was no appreciable loss of N in the last four units of the filter. When 10 parts of N as $NaNO_2$ were used 74 % of the sugar was oxidised in the first section and at the same time 1·2 parts of nitrogen as nitrate were found in the effluent. The nitrogen immobilised by the film or removed from solution in Section 1 by any other means amounted to 5·2 parts. When nitrate was used as the source of nitrogen the greatest removal of this element from the solution again occurred in Section 1, as was the case with nitrite. In Series 1, 3·5 parts N or 70 % of the total was unaccounted for in the effluent from the first section, while the corresponding figures for Series 2 were 4·3 and 43 %. The greatest utilisation of nitrate and nitrite is thus seen to occur in that part of the filter giving the highest oxidation of sugar. It is well known that micro-organisms are able to make use of oxygen in the combined state under anaerobic conditions and it is possible that some nitrogen disappeared from the nutrient solutions on passage through the filter owing to the de-oxygenation of nitrite and nitrate. As soon as the zone of vigorous carbohydrate oxidation is passed, *i.e.* beyond Section 1, loss of nitrogen from solution ceases and instead oxidation of nitrite to nitrate takes place in this part of the filter. As in the case of nitrite no more than traces of ammonia were found in the effluents from any of the sections when nitrate was used.

The results obtained with ammonium chloride show the ease of oxidation of sugar in a percolating filter, as compared with that of nitrogen. When the source of nitrogen was changed from nitrate to ammonia the filter was evidently well inoculated with organisms able to oxidise nitrite to nitrate, as shown by the results described in the previous paragraph, owing to the occurrence of nitrite in the nutrient solutions or in the effluents for a period of 121 days. However, during this period the filter had not received any appreciable quantity of ammonia. Hence the establishment of nitrite-forming micro-organisms was slow. The development of these organisms as the filter grew older and continued to receive a supply of ammonia is seen from the amounts of derived nitrate present in the final effluents in Table I C, Series 1 and 2, an average of 0·78 part and 4·16 parts respectively being obtained. Between the 122nd and 177th day of the experiment, when ammonia was added, the concentration of nitrogen as nitrite never exceeded 0·02 part per 100,000 of solution.

Increase of rate of flow from 46 g.y.d. to 186 g.y.d. The average amount of sugar oxidised by the upper one-third of the filter during the first 177 days was equal to 92·7 %. In order to extend the range in the filter of the decomposition of the carbohydrate so that sugar still appeared in the effluent from the fifth section but hardly any from the sixth, the rate of flow was increased by stages until it was four times the original rate. The effect of the greater rates of flow on the removal of carbohydrate is seen in Table II, which shows that at rates of 46, 92 and 184 g.y.d. the amounts of sugar decomposed by the first section were 92, 86 and 47 % respectively. A few tests were made at rates of 250 g.y.d. and even at this high rate of feed the oxidation of sugar effected by the whole filter amounted to over 90 %. It is interesting to note that the nitrogen present as nitrite and nitrate in the final section was 4·2 parts at 46 g.y.d. Later, when the filter was older, and presumably richer in nitrifying organisms, the increase in flow to 92 g.y.d. did not affect the time of contact sufficiently to lower the concentration of nitrite and nitrate in the effluent. On

Table II. *Effect of increase in rate of flow of sugar solution on the amount of sugar and nitrogen oxidised. Figures for sugar and nitrogen are given in parts per 100,000.*

	Rate = 46 g.y.d. Solution filtered from 141st–155th day = 100 parts sugar *plus* 10 parts N as NH_4Cl Analysis on March 18th					Rate = 92 g.y.d. Solution filtered from 156th–177th day = 100 parts sugar *plus* 10 parts N as NH_4Cl Analysis on April 9th				
	Total sugars calc. as glucose	Invert sugar calc. as glucose	Nitrogen as ammonia	Nitrogen as nitrite and nitrate	p_H	Total sugars calc. as glucose	Invert sugar calc. as glucose	Nitrogen as ammonia	Nitrogen as nitrite and nitrate	p_H
Nutrient solution	95·8	—	10·00	—	6·1	99·5	—	10·00	—	6·4
Effluent of Section 1	7·6	3·4	8·55	0·0	6·2	14·2	8·6	8·24	0·00	6·2
Effluent of Section 2	0·9	0·3	6·05	0·16	6·8	8·2	8·4	8·09	0·00	6·2
Effluent of Section 3	0·5	0·0	4·93	0·67	7·0	5·2	3·7	7·30	0·16	6·5
Effluent of Section 4	0·5	0·0	3·87	1·74	7·2	2·3	1·8	6·47	1·02	6·6
Effluent of Section 5	0·3	0·0	2·30	3·41	7·2	1·1	0·4	5·15	2·29	6·6
Effluent of Section 6	0·2	0·0	1·46	4·18	7·2	0·7	0·2	3·25	6·25	6·6

	Rate = 184 g.y.d. Solution filtered from 178th–182nd day = 100 parts sugar *plus* 10 parts N as NH_4Cl Analysis on April 14th				
	Total sugars calc. as glucose	Invert sugar calc. as glucose	Nitrogen as ammonia	Nitrogen as nitrite and nitrate	p_H
Nutrient solution	104·5	—	10·00	—	5·0
Effluent of Section 1	55·3	5·4	8·51	0·00	5·1
Effluent of Section 2	25·4	8·2	8·30	0·00	5·2
Effluent of Section 3	11·1	8·4	7·92	Trace	6·0
Effluent of Section 4	4·8	5·0	7·45	0·60	6·0
Effluent of Section 5	2·0	1·3	6·85	1·09	6·4
Effluent of Section 6	0·4	0·2	5·99	1·48	6·3

the contrary this increased to 6·3 parts N as nitrite and nitrate. A further rise in the rate to 184 g.y.d. depressed the amount of nitrite and nitrate produced in relation to the carbohydrate oxidised, probably because at this rate of flow the time of contact of solution with the organic film was less than the optimum required to oxidise ammonia.

Exp. 2.

After it had been shown that a 0·1 % solution of sugar could be almost completely oxidised in the percolating filter at a rate of 184 g.y.d. this rate was chosen for a second experiment in which the solutions were filtered in the following order:

From 178th to 196th day: 10 parts N as NH_4Cl per 100,000 of solution
197th to 209th day: 5 ,, ,, ,,
210th to 261st day: 10 ,, $NaNO_3$,,
262nd to 279th day: 5 ,, ,, ,,
280th to 296th day: 10 ,, $NaNO_2$,,
297th to 302nd day: 5 ,, ,, ,,

Results of Exp. 2.

(Table III shows the results obtained in this experiment.)

Table III. *Composition of solution and of effluents from different sections of the filter: parts per 100,000. Nutrient solution filtered: sucrose nominally equal to 105·3 parts glucose.*

Rate of filtration = 184 g.y.d.

A. Nitrogen added as ammonium chloride	Series 1 (178th to 196th day). Average results of 3 sets of analyses made on the 182nd, 189th and 196th day. Amount of nitrogen added = 10 parts per 100,000. C:N ratio = 4·2:1							Series 2 (197th to 209th day). Average results of 2 sets of analyses made on the 202nd and 209th day. Amount of nitrogen added = 5 parts per 100,000. C:N ratio = 8·4:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugars (calc. as glucose)	106·0	51·6	20·0	10·7	5·0	2·2	0·5	109·4	59·0	27·4	11·1	6·1	2·6	0·9
Sucrose (calc. as glucose)	106·0	43·8	15·3	4·8	1·5	1·1	0·3	109·4	53·9	21·6	4·7	1·3	0·5	0·4
Invert sugar (calc. as glucose)	—	7·8	4·7	5·9	3·5	1·1	0·2	—	5·1	5·8	6·4	4·8	2·1	0·5
Nitrogen as ammonia	10·00	8·71	8·19	8·16	7·92	6·83	6·26	5·00	3·75	3·99	3·82	3·65	3·55	2·63
Nitrogen as nitrite	—	0·00	0·00	0·00	0·00	0·00	0·00	—	0·00	0·00	0·00	0·00	0·00	0·00
Nitrogen as nitrate	—	0·00	0·00	0·00	0·29	0·38	1·30	—	0·00	0·00	0·00	0·00	Trace	0·66
Total inorganic nitrogen	10·00	8·71	8·19	8·16	8·21	7·21	7·56	5·00	3·75	3·99	3·82	3·65	3·55	3·29
P_H (one determination)	5·0	5·1	5·2	6·0	6·0	6·4	6·3	—	—	—	—	—	—	—

B. Nitrogen added as sodium nitrate	Series 1 (210th to 261st day). Average results of 5 sets of analyses made on the 216th, 218th, 239th, 251st and 261st day. Amount of nitrogen added = 10 parts per 100,000. C:N ratio = 4·2:1							Series 2 (262nd to 279th day). Average results of 2 sets of analyses made on the 267th and 273rd day. Amount of nitrogen added = 5 parts per 100,000. C:N ratio = 8·4:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugars (calc. as glucose)	103·0	58·6	34·3	23·8	16·4	10·0	5·8	96·1	59·0	30·1	16·6	12·0	4·8	0·8
Sucrose (calc. as glucose)	99·8	50·3	24·7	13·3	5·7	2·5	1·5	94·4	54·9	25·6	13·2	10·0	3·9	0·4
Invert sugar (calc. as glucose)	3·2	8·3	9·6	10·5	10·7	7·5	4·3	1·7	4·1	4·5	3·4	2·0	0·9	0·4
Nitrogen as ammonia	0·01	0·05	0·08	0·14	0·14	0·15	0·18	0·03	0·02	0·02	0·01	0·01	0·02	0·02
Nitrogen as nitrite	0·01	0·86	0·91	0·91	0·88	0·72	0·68	0·53	0·43	0·50	0·55	0·50	0·55	0·29
Nitrogen as nitrate	9·98	5·98	4·34	3·43	3·25	2·82	2·60	4·44	1·41	1·15	0·73	0·52	0·47	0·32
Total inorganic nitrogen	10·00	6·89	5·33	4·48	4·27	3·69	3·46	5·00	1·86	1·67	1·29	1·03	1·04	0·63
P_H	5·9	6·1	6·3	6·3	6·6	6·9	7·0	5·6	6·8	6·6	6·8	6·8	7·0	7·1

Sugar oxidation. Since on statistical examination all the six sets of results do not show any significant difference it may be concluded that sufficient nitrogen is provided for the organisms in the filter with a C:N ratio of 8·4:1 or less. There is ample evidence [unpublished work of Dawson, Jenkins and Martin] and Barritt [1931, 1] that carbohydrate solutions of C:N ratio greater than 8·4:1 can be oxidised efficiently on percolating filters. Table III also

Table III (*cont.*)

C. Nitrogen added as sodium nitrite	Series 1 (280th to 296th day) Average results of 5 sets of analyses made on the 283rd, 288th, 290th, 293rd and 296th day Amount of nitrogen added = 10 parts per 100,000 C:N ratio = 4·2:1							Series 2 (297th to 302nd day) Average results of 2 sets of analyses made on the 300th and 302nd day Amount of nitrogen added = 5 parts per 100,000 C:N ratio = 8·4:1						
	Nutrient solution	Section 1	2	3	4	5	6	Nutrient solution	Section 1	2	3	4	5	6
Total sugar (calc. as glucose)	103·3	43·1	17·9	10·0	4·6	1·2	0·5	106·9	66·0	34·2	15·9	4·9	1·3	0·6
Sucrose (calc. as glucose)	101·5	36·9	13·3	7·0	3·4	0·9	0·2	106·2	59·4	27·3	12·8	3·6	0·0	0·3
Invert sugar (calc. as glucose)	1·8	6·2	4·6	3·0	1·2	0·3	0·3	0·7	6·6	6·9	3·1	1·3	1·3	0·3
Nitrogen as ammonia	0·02	0·02	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
Nitrogen as nitrite	9·98	5·47	2·43	1·33	0·81	0·51	0·27	5·00	2·55	0·70	0·11	0·01	0·00	0·00
Nitrogen as nitrate	0·00	0·26	0·24	0·48	0·72	0·72	0·84	0·00	0·04	0·63	0·13	0·24	0·07	0·13
Total inorganic nitrogen	10·00	5·75	2·67	1·81	1·53	1·23	1·11	5·00	2·59	1·33	0·24	0·25	0·07	0·13
P_H	6·5	6·9	6·8	6·9	7·0	7·2	7·3	6·5	6·6	6·6	6·6	6·7	6·9	7·0

proves that when the C:N ratio provides enough available nitrogen for micro-biological requirements under the conditions of the experiment it does not matter whether the nitrogen is supplied as nitrite, nitrate or ammonia. A supply of nitrogen in excess of the needs of the film has no measurable effect on the oxidation of sugar.

The proportion of invert sugar in the total sugar contained in the effluents from the sections in Exp. 2 was considerably less than in the corresponding effluents during Exp. 1. The results of the latter experiment showed the highest percentage of invert sugar to be present in the effluents of the upper sections when nitrite was used, *i.e.* at the start of the experiment. As the filter grew older the amount of invert sugar in solution became less, falling from 32·2 parts in Section 1, Table I A, Series 1, to 6·2 parts in Section 1, Table I C, Series 2, and to 6·6 parts in Section 1, Table III C, Series 2. Hence it appears that the presence of large quantities of invert sugar in the effluents of a percolating filter is indicative of a state of immaturity.

Nitrogen results. The absence of nitrite from all the effluents obtained when ammonium chloride was supplied at the high rate of flow in Exp. 2 and the small amount of nitrate produced support the view previously advanced in this paper that the oxidation of nitrogen is sensitive to changes in rate of flow or time of contact. Nitrate did not appear until the fourth section had been reached when 10 parts of ammonia-nitrogen were supplied while only 0·66 part N as NO_3 was obtained in the last section when 5 parts were added. Including nitrogen immobilised by the micro-organisms, an average of 30 % of the nitrogen fed as ammonium chloride was unaccounted for in the final effluents.

The results shown in Table III B resemble those obtained with sugar and nitrate at a rate of 46 g.y.d. (Table I B) in that nitrite was found in the effluents from all sections. At the lower rate of flow, however, the reduction of nitrate to nitrite was most vigorous in the first section of the filter where carbohydrate oxidation was also greatest. In both series of Exp. 2 the amounts of nitrogen removed from the solutions by Section 1 were significantly lower when am-

monium chloride was the source of nitrogen than was the case when either sodium nitrite or sodium nitrate was supplied. Thus the percentages of nitrogen unaccounted for in the effluent of Section 1 when 10 and 5 parts respectively of ammonium chloride were present amounted to 13 and 25 respectively, the corresponding figures for nitrite being 43 and 48, and for nitrate 31 and 63. These figures indicate that either (1) more nitrogen is used to build up the film in a filter when nitrite or nitrate is supplied than if ammonia is used or (2) that nitrite and nitrate play a part in the oxidation of sugar which ammonia does not. If there is any truth in the latter speculation the additional rôle of nitrite and nitrate as suppliers of oxygen for the combustion of sugar did not result in any significant difference in the amount of carbohydrate oxidised by all the three forms of nitrogen used. The apparent loss of nitrogen in Exp. 2 was considerable, an average of 76 % being recorded during the period of 70 days when nitrogen was provided as sodium nitrate.

A large proportion of the nitrite supplied from the 280th to 296th day could not be accounted for in the effluents. Only 58 % was recovered in the effluent of Section 1 and this figure fell to 11 % in the final effluent. At this stage of the experiment there did not appear to be the increase in the amount of film produced which would have been expected if there had been a quantitative conversion into microbial tissue of all the nitrogen unaccounted for in the effluents. In all the sections nitrate was found, so that oxidation of carbohydrate and of nitrite had occurred simultaneously. The amount of inorganic nitrogen which was not recovered in the final effluents reached an average figure of 94 % for the 23 days of this part of the experiment.

Discussion of results.

It is a well-established fact that micro-organisms require nitrogen for their growth. The result of the two experiments which have been described in this paper show that in oxidising sugar by biological filtration the organisms concerned are able to use nitrogen as ammonium chloride, sodium nitrite or sodium nitrate with equal efficiency.

As yet there does not appear to be any means of determining the availability of a nitrogenous compound under the conditions of a biological filter other than the tedious one of putting the compound through a prolonged filtration test. Degrees of availability of different nitrogenous substances may exist; in connection with the decomposition of plant materials Richards and Norman [1931] state that, "Plant materials already containing sufficient or more than sufficient nitrogen for decomposition may nevertheless immobilise an additional amount, owing to preferential utilisation of the inorganic form." The availability of organic sources of nitrogen such as fungal tissues has been studied by Norman [1933] and compared with the availability of various inorganic sources of nitrogen. Organic forms of nitrogen may be assimilated by organisms just as readily as inorganic forms and, as in the case of albumin [Jenkins, unpublished work] may actually possess certain advantages over, say, ammonium salts by increasing the time of contact of a new filter. For practical reasons, however, nitrogen is best supplied as ammonia since in this form relatively little loss of nitrogen appears to result. Other nitrogenous compounds, such as those present in the effluents from the pulp presses of beet-sugar factories seem to be only slowly decomposed and temporarily immobilised before being utilised by micro-organisms.

In a previous paper [Jenkins, 1931] the author described experiments with a filter of fixed sections separated by air spaces and found that the maximum amount of invert sugar obtained from 100 parts of sucrose was 15 parts in the effluent of Section 4. When this filter reached its maximum efficiency some of the effluents contained nearly 11 parts of invert sugar. The results obtained in the present work, however, suggest that the products of inversion appear in large amounts in a new filter but do not persist except in comparatively small amounts in a mature filter. Compared with the improved wooden sectional filter used for this work the fixed sectional filter was inefficient, as it could only oxidise considerably less than one-half the amount of sugar oxidised by the former.

The p_H figures of the effluents which were recorded during these experiments showed that there was generally no measurable production of acid on filtration. The only reductions of p_H observed during both experiments were small and possibly insignificant in amount, *viz.* from 6·4 to 6·2 in Table I C, from 6·4 to 6·2 and 6·4 to 6·3 in Table II, from 6·4 to 6·3 in Table III A, from 6·8 to 6·6 in Table III B, and from 6·9 to 6·8 in Table III C.

The nutrient solutions, as previously mentioned, were made up daily in distilled water. Since the aspirators in which the solutions were kept occasionally contained microbial growths, in spite of daily cleaning, inversion of the sugar sometimes occurred to a small extent. This inversion could be ignored when compared with the amount of hydrolysis of the sugar on filtration. As much as 50 % of the sugar in the effluents from Sections 1 and 2 was present in the invert form when the filter was immature. Later, when the efficiency of the film increased, less invert and less total sugar were found. It is of interest to know that a young film may be so active in effecting the first stage of carbohydrate degradation, *viz.* hydrolysis.

In a filter able to oxidise completely the sucrose presented to it the ratio of invert to total sugar in any section appears to be small, presumably because the invert sugar is then decomposed as rapidly as it is formed. Maximum efficiency in a percolating filter is reached as the filter ages. In this respect the conditions in a filter differ from those which exist in a pure culture of organisms, where biological activity falls off with the age of the culture. The population of a biological filter becomes more varied with continued operation. Although the flora of the film rarely remains the same over a long period [unpublished work of Cutler, Crump, Dixon and Sandon] it is believed that any changes in its biological composition are in the direction of a more varied and specialised population, each organism specialising in those reactions which it is able to carry out in the presence of other competing organisms.

In the early stages of development a filter requires an ample supply of nitrogen, besides other elements, for the growth of its population. Once an active film has been established nitrogen is still required, but in smaller quantities. It is in fact only necessary to replace the losses of nitrogen which are known to occur either through denitrification or by the removal of dead organisms. The results given in the Tables point to losses of nitrogen far in excess of the quantity which would be required to maintain the growth of the film. The disappearance of nitrogen is seen to be most marked in the case of sugar solutions which contained nitrite or nitrate. For instance in Table III C, Series 1, 10 parts of nitrogen per 100,000 were reduced to 5·75 parts in the first section of the filter. This is equivalent to a removal of 0·021 % $NaNO_2$ from a solution originally containing 0·049 %. Now it is possible by physical methods to determine the average time during which the solution is in contact with

the film [Clifford, 1907]. Measurements of the time of contact on the filter under discussion have not been made, but from numerous experiments carried out with percolating filters filled with the same size of clinker it is estimated that at a rate of 184 g.y.d. the average time of passage of the nutrient solution through the first section would be between $\frac{3}{4}$ and 1 hour. In the time taken to percolate through the six sections of the filter, say about 4 hours[1], 0·044 % of the sodium nitrite in the solution originally containing 0·049 % had been decomposed. At first sight it appears that this rapid denitrification, which occurs immediately the nutrient solution is run on to the filter, conflicts with the results of Lloyd and Cranston [1930]. These workers found that under anaerobic conditions in pure culture a denitrifying marine organism isolated from Loch Striven completely denitrified a 0·313 % solution of KNO_2 in about 60 hours. However, there was an initial lag phase of 18 hours before gas production occurred. Under natural conditions, when the organisms were developing normally this lag phase would be reduced or disappear entirely. Moreover, it is not at all unlikely that the rate of evolution of gas would be increased in a natural environment owing to the stimulating action of other organisms [Cutler and Crump, 1929; Meiklejohn, 1930]. As previously mentioned a considerable quantity of nitrogen was unaccounted for in the effluents when solutions of ammonium chloride and sugar were filtered. Although the amount which disappeared in this case was less than the losses with nitrite and nitrate it is possible that the continuous removal of large amounts of ammonia from solution might be due to some loss of gaseous nitrogen in addition to the nitrogen taken up by the film. The data given in this paper, however, cannot be taken as proof of the loss of nitrogen in the free state.

The causes of denitrification under varying experimental conditions have been studied by several workers. Many soil bacteria such as *Bact. mycoides* [Marchal, 1893] are able to transform nitrates into ammonia with nitrites as intermediate compounds, while others can only reduce nitrates to free nitrogen. Muntz and Lainé [1911] found that denitrification occurs in percolating filters when readily oxidisable organic matter and nitrates are treated on such filters. These workers observed a loss of 60 % nitrate in several experiments. A nitrogen balance sheet of the bio-aeration method of sewage purification was drawn up by Richards and Sawyer [1922] who attributed losses of nitrogen in the process to the simultaneous presence of organic matter and oxidised forms of nitrogen. Although it has been believed that both amino-acids and nitrites lose free nitrogen when they interact in the presence of micro-organisms, Barritt [1931, 2] has shown that it is only the nitrite which suffers denitrification. There is no experimental evidence to support the view sometimes put forward [Waksman, 1931] that denitrification under natural conditions may result from the union of ammonia and nitrous acid, followed by the decomposition of the ammonium nitrite thus formed into nitrogen and water. The results obtained with the Scott-Moncrieff [1909] sectional biological filter do not support this opinion.

The results given in this paper agree with those found by other workers in showing that denitrification is most vigorous in the presence of decomposable organic matter. Decomposition of nitrite in a percolating filter is not inhibited by the presence of oxygen. In the present experiments the solutions in which denitrification was active contained between 0·4 and 0·6 part per 100,000 of dissolved oxygen and being spread over the surface of the clinker in thin films

[1] The time of percolation through six sections would not be six times that of the first section because the large quantity of film in this section offers a relatively greater resistance to the passage of liquid. Section 1 would thus give a longer time of contact than any other part of the filter.

the liquids were constantly exposed to a supply of air. Oxygen tension has a greater effect in arresting nitrate reduction in pure culture than in percolating filters. For instance Stickland [1931] found that *Bact. coli* reduces nitrates quantitatively to nitrite under anaerobic conditions. This investigator found that it required only 3·8 % saturation of dissolved oxygen in the culture medium to cause a 93 % decrease of this reduction, whereas in the experiments with percolating filters described in this paper vigorous reduction occurred when the oxygen tension was as high as 60 % of saturation. The extent of the loss of nitrogen depends mainly upon the C:N ratio but also upon the type of nitrogen compound. If this ratio is large and nitrogen compounds other than nitrates or nitrites are absent, the nitrogen supplied may be used entirely for structural purposes and so locked up in the filter. With a small C:N ratio some nitrogen may remain unaccounted for, even when due allowance has been made for microbiological requirements; this is especially likely if the source of nitrogen is nitrite or nitrate.

Unpublished work by Dawson, Jenkins and Watkins has shown that a 0·1 % solution of sucrose may be completely oxidised on percolating filters if a C:N ratio of about 25:1 is maintained. This agrees reasonably well with the ratio of 15:1 which Barritt [1931, 1] considers necessary for 0·2 % sucrose solutions. Under the conditions of experiment described in this paper the same results for sugar oxidation were obtained with C:N ratios of 4·2:1 as with 8·4:1. Obviously both these ratios supply more nitrogen than is required to obtain the maximum oxidation of sugar. It is improbable that any greater rate of purification of organic matter can be expected by decreasing a C:N ratio already providing a large proportion of available nitrogen.

Summary.

1. A percolating filter of new design was used to compare the extent of sucrose oxidation in the presence of ammonium chloride, sodium nitrite and sodium nitrate. The differences in degree of oxidation were not significant and it is concluded that none of the forms of nitrogen has any outstanding advantage as far as sugar oxidation is concerned. With all three compounds of nitrogen the filter oxidised 28 litres of 0·1 % sucrose solution every day for 4 months (equivalent to a rate of 184 gallons per cubic yard per day (g.y.d.).

2. If the ratio of invert sugar to sucrose in the effluents of a filter receiving sucrose is high, it is probable that the filter is immature or that it is not working up to its capacity.

3. Under the conditions of filtration in these experiments there was a considerable disappearance of nitrogen from solutions having C:N ratios of 8·4:1 and 4·2:1, irrespective of the form in which the nitrogen was supplied. The greatest disappearance of nitrogen occurred with nitrite, when 97 % was removed from solution. The least occurred with ammonia-nitrogen, when 24 % was removed from solution. The apparent loss of nitrogen supplied as nitrite and nitrate and, to a smaller extent as ammonium chloride, was most marked in that part of the filter where carbohydrate oxidation was most active. These figures for apparent losses of nitrogen include the nitrogen immobilised by the film and without further experiment it is impossible to state how much, if any, of the nitrogen is actually lost as elementary nitrogen from the system.

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Scientific and Industrial Research and the results are published by permission of the Department. The work was supervised by Mr E. H. Richards to whom the author is indebted for encouragement and criticism. Thanks are due to Miss Crump, Miss Dixon, Messrs Cutler, Dawson, Martin, Sandon and Watkins whose unpublished results have been mentioned.

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XXXVIII. THE BIOLOGICAL OXIDATION OF CARBOHYDRATE SOLUTIONS.

III. NITROGEN, PHOSPHORUS AND POTASSIUM BALANCES IN PERCOLATING FILTERS.

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The liberation of elementary nitrogen from its compounds is a process which has been observed in many biological environments. It is reported to occur in soils rich in nitrogenous organic substances and especially in tropical soils [Meggitt, 1923–25]. Manure heaps are also known to evolve free nitrogen in addition to large amounts of ammonia [Pfeiffer *et al.* 1897]. Similar losses of gaseous nitrogen have been shown by Richards and Sawyer [1922] to occur in the activated sludge or bio-aeration process for purifying sewage. Muntz and Lainé [1911] drew up a balance sheet for nitrogen in a mixture of sewage and nitrate and recorded considerable losses of nitrogen. The only evidence which exists to point to a similar phenomenon in percolating filters is purely circumstantial. Adeney and Letts [1908] found the concentration of nitrogen in the effluent from a percolating filter to be greater than in the sewage before treatment. The addition of nitrate to sewage followed by filtration through soil [Muntz and Lainé, 1911] resulted in a loss of nitrogen from the solution considerably in excess of the amount retained by the soil. A percolating filter fed with a solution of sucrose having a C:N ratio of 100:1 was found by Barritt [1931, 1] to gain about 30% of nitrogen. The solutions used by this author were made up in Harpenden tap-water, which contains a variable amount of nitrogen as nitrate. Thus Barritt found 8 parts of nitrogen as nitrate in one million parts of water while at a different time the author found the water to contain 5 parts of nitrogen as nitrate and from 5 to 7 parts in the organic form. However, even when allowance is made for these variations in the composition of the diluting water, the gain in nitrogen is quite appreciable and agrees with the conclusions of several workers [Hutchinson and Richards, 1921; Hutchinson, 1918–19] that soils and fermenting carbohydrates fix nitrogen most actively under conditions of nitrogen starvation. The author observed in previous experiments [Jenkins, 1933, 2] that under certain conditions nitrogen was apparently lost from solution during biological filtration whether ammonia, nitrite or nitrate were the source of nitrogen. The object of the experiments described in this paper was primarily to check this conclusion by drawing up balance sheets for nitrogen from the data yielded by filters operated under controlled conditions. Secondary objects were to find the phosphate and potash requirements of the biological film and the ratio of carbon oxidised to carbon compounds synthesised and the loss of moisture by evaporation during filtration.

A special technique was required to ensure freedom from available forms of nitrogen, phosphorus and potassium in the filter medium and for this purpose sectional glass filters and glass media were used. The constructional details of these experimental filters have been described elsewhere [Jenkins, 1933, 1]. The medium employed in Exp. 1 differed from that used in Exp. 2, while in Exp. 3 the filters used were of a semi-commercial size and the liquids filtered consisted of effluents from a beet-sugar factory. These three experiments are therefore considered separately.

Exp. 1. The filter consisted of 6 glass sections, 7″ deep and $3\frac{1}{4}$″ in diameter, containing $\frac{1}{4}$″ to $\frac{1}{16}$″ graded glass made from broken bottles.

Solution filtered:		
	Sucrose	1 g.
	$MgSO_4, 7H_2O$	0·0131 g.
	$CaCl_2, 6H_2O$	0·0131 g.
	NaCl	0·0013 g.
	P_2O_5 (as K_2HPO_4)	0·0300 g.
	K_2O (as K_2HPO_4)	0·0398 g.
	N as NH_4Cl	0·0500 g.
	Distilled water	1 litre
	C:N ratio	8·4:1
	p_H	6·0

Two litres of this solution were made up daily and run on to the filter at a rate of 100 gallons of solution per cubic yard of filtering material per day (abbreviated g.y.d.) for a period of 108 days. Additional K_2O as K_2SO_4 was supplied from the 21st to the 66th day, bringing the total K_2O to 0·0668 g. per litre. This additional K_2O was supplied to ensure an excess of potash. When the quantity given in the first place was found to be ample the addition of the extra K_2O was discontinued. Until the 66th day of the experiment the source of N was NH_4Cl; later NH_4HCO_3 was used. In glass filters none of the free acid liberated by the removal of NH_3 from the ammonium salt is neutralised, and as a result the p_H value with NH_4Cl may fall to 3·0 and check the development of organisms in the lower sections of the filter. This drop in p_H has been proved to be due chiefly, if not entirely, to the liberation of mineral acid, since the p_H of the solution undergoing treatment was never lowered when the source of nitrogen was NH_4HCO_3.

Records were kept of the total volume filtered daily and the volume of effluent obtained. At the end of the experiment, when the filters were dismantled for analysis of the film, the quantity of water retained by the medium and film was determined. Representative samples of effluent were collected and analysed each day for sugar and each week for nitrogen, phosphorus and potash. Tests were also carried out for nitrite and nitrate, while approximate measurements of the dissolved organic matter were made by the test for "biochemical oxygen demand" in 5 days [Ministry of Health, 1929]. The methods of analysis were similar to those previously described [Jenkins, 1933, 2]. The film adhering to the medium was washed off at the conclusion of the experiment and analysed immediately for organic matter, N, P and K. Nitrogen was determined by the Kjeldahl method; phosphorus was determined by digesting the concentrated solution of effluent as in the Kjeldahl method, precipitating the phosphorus as magnesium ammonium phosphate and then weighing the phosphorus as $Mg_2P_2O_7$: potassium was estimated by the perchlorate method.

The amounts of sugar oxidised expressed as percentages of the quantity supplied are given graphically in Fig. 1 and show that complete oxidation was

being achieved after the filter had been in operation for 20 days. After the 55th day, however, film growth prevented the free passage of solution and air through the filter, which could not be operated continuously without pools forming on the surface. Balance sheets for volume of liquid and weight of nitrogen, phosphorus and potassium were drawn up and the results are given in Tables I–IV. It is noteworthy that less than 2% of the solution was lost by evaporation over a period of 108 days; this result is confirmed by later work (see Exp. 2).

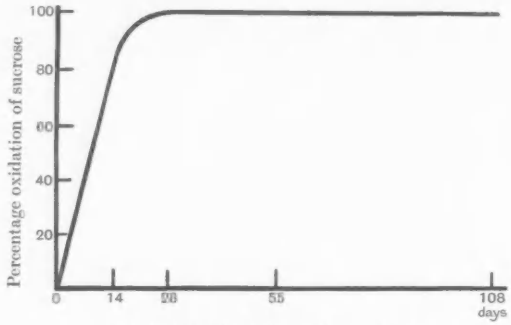

Fig. 1. Oxidation of sugar in Exp. 1.

Table I. *Preliminary experiment with C:N ratio = 8·4:1. Volume balance sheet (Exp. 1).*

Period days	Volume filtered cc.	Volume recovered as effluent cc.	Volume retained by filter *plus* vol. lost by evaporation cc.
0–6	9,913	9,765	148
7–13	11,087	10,882	205
14–20	11,470	11,231	239
21–27	12,123	11,964	159
28–34	11,284	11,118	166
35–41	11,050	10,872	178
42–48	9,413	9,188	225
49–55	8,958	8,711	247
56–69	10,088	9,945	143
70–84	12,569	12,236	333
85–108	11,682	11,249	433
Total	119,637	117,161	2476

Liquid in filter = 352
Loss by evaporation = 2124 = 1·8 %

The figures for nitrogen show a loss of 14·2% of the total supplied. Since neither nitrite nor nitrate formation in the filter was observed during the experiment, and the heavy growth of film rendered such oxidation of ammonia most unlikely, it is improbable that the nitrogen lost was caused by the formation of nitrite or nitrate, followed by the interaction of either compound with organic matter and the subsequent liberation of gaseous nitrogen. Moreover, as the liquids filtered were always acid (p_H 6·0 or less) the loss of over 14% of nitrogen could not have resulted from the volatilisation of ammonia. It will be observed that the film in the third section contained more nitrogen than that in the second section. The amount of film is usually greatest in the top of the filter and decreases in quantity to the bottom. A steady decrease in amount of film

Table II. *Preliminary experiment with C:N ratio = 8·4:1. Nitrogen balance sheet (Exp. 1).*

Period days	Nitrogen added as NH_4Cl or NH_4HCO_3 g.	Nitrogen recovered in effluent, g. Total N	NH_3-N	Organic N
0–6	0·4956	0·4328	0·4221	0·0107
7–13	0·5544	0·1840	0·1411	0·0429
14–20	0·5735	0·2327	0·2008	0·0319
21–27	0·6061	0·4218	0·3999	0·0219
28–34	0·5642	0·5132	0·5005	0·0127
35–41	0·5525	0·2368	0·2306	0·0062
42–48	0·4707	0·2103	0·1976	0·0127
49–55	0·4479	0·3056	0·2187	0·0869
56–69	0·5044	0·4925	0·4175	0·0750
70–84	0·6284	0·6890	0·5570	0·1320
85–108	0·5841	0·6415	0·5575	0·0840
Total	5·9818	4·3603	3·8433	0·5180

Section No.	Nitrogen recovered in film, g. Total N	NH_3-N	Organic N
1	0·3464	0·0536	0·2928
2	0·0635	0·0158	0·0477
3	0·1433	0·0294	0·1139
4	0·0728	0·0163	0·0565
5	0·0711	0·0147	0·0564
6	0·0759	0·0209	0·0550
Total	0·7730	0·1507	0·6223

Nitrogen recovered = 4·3603 + 0·7730 = 5·1333 g.
Nitrogen unaccounted for = 5·9818 − 5·1333 = 0·8485 = 14·2 %

Table III. *Preliminary experiments with C:N ratio = 8·4:1. Phosphorus balance sheet (Exp. 1).*

Period days	P_2O_5 added in nutrient solution g.	P_2O_5 recovered in effluent g.
0–6	0·2974	0·2226
7–13	0·3326	0·1498
14–20	0·3441	0·1325
21–27	0·3637	0·2327
28–34	0·3385	0·3048
35–41	0·3315	0·1906
42–48	0·2824	0·1875
49–55	0·2687	0·1778
56–69	0·3026	0·2701
70–84	0·3771	0·4184
85–108	0·3505	0·4375
Total	3·5891	2·7243

Section No.	P_2O_5 recovered in film g.
1	0·1413
2	0·0274
3	0·0250
4	0·0144
5	0·0517
6	0·0177
Total	0·2775

P_2O_5 recovered = 2·7243 + 0·2775 = 3·0018 g.
P_2O_5 unaccounted for = 3·5891 − 3·0018 = 0·5873 g.

Table IV. *Preliminary experiments with C:N ratio = 8·4:1. Potash balance sheet (Exp. 1).*

Period days	K_2O added in nutrient solution g.	K_2O recovered in effluent g.
0–6	0·3954	0·3913
7–13	0·4412	0·3743
14–20	0·4564	0·3244
21–27	0·8099	0·6142
28–34	0·7538	0·8110
35–41	0·7381	0·6818
42–48	0·6288	0·5799
49–55	0·5984	0·4358
56–69	0·5852	0·3908
70–84	0·5002	0·6534
85–108	0·4659	0·4427
Total	6·3714	5·6996

Section No.	K_2O recovered in film g.
1	0·0610
2	0·0132
3	0·0206
4	0·0097
5	0·0108
6	0·0147
Total	0·1300

K_2O recovered = 5·6996 + 0·1300 = 5·8296 g.
K_2O unaccounted for = 6·3714 − 5·8296 = 0·5418 g.

was not found in this experiment because film which stuck on to the bottom of the basal plate of Section 2 was included with the medium in Section 3 in order to avoid loss of organic matter when dismantling the filter for analysis.

The apparent incomplete recovery of phosphorus and potash may be attributed largely to errors involved in the gravimetric methods of analysis employed for the small quantities of these substances. The results for the amounts contained in the effluents and in the film should probably be about 10 % greater than the figure given. The actual results of analysis showed that the film contained 2·3 % P_2O_5 and 1·1 % K_2O on the dry weight since 11·87 g. of film were contained in the whole filter.

Exp. 2. The grade of medium used in Exp. 1 was evidently too small as ponding of liquid on the top surface of the filter occurred in the later periods of the experiment. In some new filters which were constructed [Jenkins, 1933, 1] short lengths of glass tubing 1 cm. diameter by 1·5 cm. length were found to be more satisfactory as filtering medium than the graded glass from bottles used in Exp. 1. Filters were accordingly filled with short lengths of glass tubing for Exp. 2. This experiment was begun before the conclusion of the previous experiment. The objects of Exp. 2 were the same as those of Exp. 1, *viz.* to draw up a balance sheet for the amount of nitrogen supplied to a percolating filter and the amount recovered in the film and in the effluents, and also to obtain similar balances of phosphorus and potassium. In Exp. 2, however, two filters were used and these were supplied with solutions of sugar and ammonium bicarbonate so as to provide C:N ratios of 8·4:1 and 84:1 respectively. The details of the experiment are described below. The filters used each consisted of four glass cylinders, 18 cm. long and 8 cm. in diameter containing the glass tubes. They were fed daily with 2 litres of the following solution, *i.e.* at 100 g.y.d.

	Filter A C:N ratio = 8·4:1	Filter B C:N ratio = 84:1
Sucrose	1 g.	1 g.
$MgSO_4, 7H_2O$	0·0131 g.	0·0131 g.
$CaCl_2, 6H_2O$	0·0131 g.	0·0131 g.
NaCl	0·0013 g.	0·0013 g.
P_2O_5 (as K_2HPO_4)	0·0300 g.	0·0300 g.
K_2O (as K_2HPO_4)	0·0398 g.	0·0398 g.
N (as NH_4HCO_3)	0·0500 g.	0·0050 g.
Distilled water	1 litre	1 litre
p_H	6·0	6·0
Experimental period	58 days	70 days

The filters were operated simultaneously and the same methods of sampling and analysis were used as in Exp. 1. Filter A was dismantled after 58 days and analyses were made immediately. Filter B was operated for 12 days longer so that the analytical periods would not overlap.

The percentages of sugar oxidised in Filters A and B are shown graphically in Fig. 2. While the former produced an almost sugar-free effluent after 12 days and continued to do so even after the rate of feed of sugar solution was doubled, Filter B was extremely erratic in the quantity of sugar it oxidised. It almost seems as if there existed a periodicity of sugar oxidation in which maximum combustion was attained every 4th or 5th day. The oscillation in efficiency of this filter was still marked but not quite so striking when the rate was increased to 200 g.y.d., although the rate of flow, composition of nutrient solution and other experimental details were the same from day to day. It is extremely improbable that the causes of the fluctuation in the amount of sugar found in the effluent from day to day were methodical errors, since the rate of feed of

the nutrient solution and the concentrations of sugar in these liquids were kept constant, while determinations of sugar were made on the whole of the effluent collected in one day. The daily variation in the percentages of sugar oxidised must therefore result from daily differences in the biological activity of the micro-organisms in the filter as regards the oxidation of sugar. In order to show that the composition of the effluent remains constant over a period of several hours when supplied with a nutrient liquid of constant composition at a steady rate of flow, sampling at frequent intervals would have been necessary. The chief object of the experiment, as has been stated previously, was to obtain a complete recovery of nitrogen in the film and effluents and as the removal of samples at short intervals of time for the determination of sugar would have

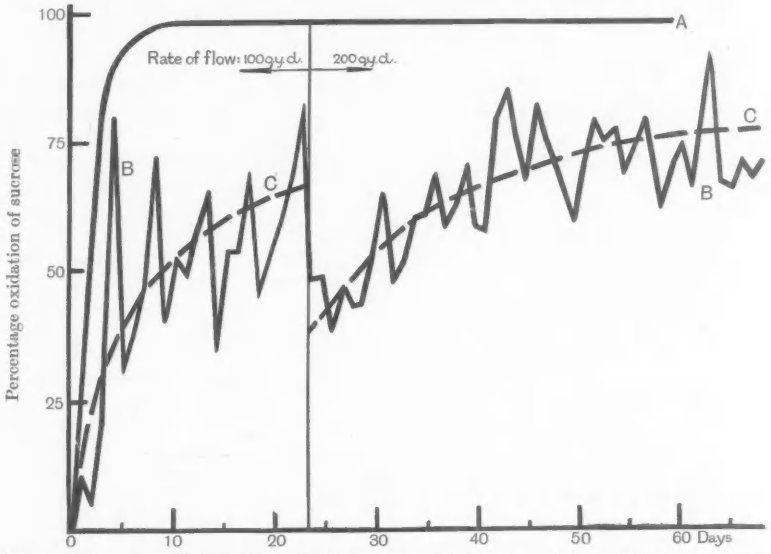

Fig. 2. Percentage oxidation of sugar by filter receiving C:N ratios of 8·4:1 and 84:1 respectively (Exp. 2). A: Filter fed with a solution of 100 parts of sucrose, 3 parts P_2O_5 (K_2HPO_4) and 5 parts N (NH_4HCO_3) per 100,000. B: Filter fed with a solution of 100 parts of sucrose, 3 parts P_2O_5 (K_2HPO_4) and 0·5 part N (NH_4HCO_3) per 100,000. C: General trend of Curve B.

resulted in removal of nitrogen such continuous sampling was not carried out in this experiment. However, in a later experiment made at Colwick, Nottingham (see Exp. 3), analyses were made at regular periods on the effluents of two filters, which received the same nutrient solution and produced consistently similar effluents. The only difference between these two filters was that one was inoculated by Mr Cutler and Miss Crump of Rothamsted with selected organisms, while this treatment was omitted from the other filter. The results of these analyses are shown in Table V.

These figures show that over a period of several hours the maximum variation of the composition of the effluent from the mean value as measured by the "4 hours" and sugar analyses was less than 10%: the p_H of the effluents varied only slightly. It is probable that the results of analysis with a laboratory filter fed with a solution of constant composition and at a steady rate of flow

Table V.

Time of sampling p.m.	p_H		Parts per 100,000			
			"4 hours"*		Sugar	
	Inoculated filter	Uninoculated filter	Inoculated	Uninoculated	Inoculated	Uninoculated
12.01–12.04	6·9	6·7	31·9	32·0	10·7	10·3
12.04–12.09	6·8	6·6	31·5	33·8	12·1	11·4
12.09–12.15	6·8	6·5	33·7	34·7	11·3	12·4
12.15–12.20	6·8	6·5	32·7	34·3	11·3	11·3
12.20–12.25	6·9	6·4	30·4	35·9	11·6	11·6
12.25–12.30	6·8	6·5	30·8	36·3	10·6	10·9
12.30–12.35	6·8	6·5	30·8	33·0	11·9	11·7
12.42–12.52	6·8	6·4	30·2	33·8	11·0	11·1
4.0	6·8	6·4	31·1	33·4	11·3	11·9
6.0	6·7	6·3	34·3	34·9	9·4	12·1

* The "4 hours" figure is a measure of the organic matter oxidisable by permanganate.

would show even smaller oscillations than those obtained with a semi-commercial plant.

The daily fluctuation in the amount of sugar oxidised in Exps. 2 B and 3 may have its counterpart in the day to day variation in bacterial numbers. Thornton and Gray [1930] showed that the numbers of bacteria in soil rarely remain static but oscillate between wide intervals in short periods. Sandon and Dixon [unpublished work] have found similar changes in the numbers of bacteria, fungi and protozoa in percolating filters, while the numbers of *Staphylococcus aureus* in meat juices were found to fluctuate over a period of 44 days [Foreman and Graham Smith, 1928]. At this stage of the experiments any explanation of the fluctuation in the actual performance of the nitrogen-starved filter can only be regarded as tentative. It is suggested that a peak in the graph represents a condition in the filter when all the incoming nitrogen, whether in the form of ammonia or as nitrogen liberated by bacterial autolysis is being used for microbial development. A large proportion of the dissolved sugar is then required by the organisms and the amount of sugar oxidised is correspondingly great. The slope from a peak down to a trough is assumed to be caused by unfavourable growth conditions in which only part of the ammoniacal form and none of the organic form of nitrogen is utilised, so that less sugar is needed by the population of the filter. Cutler *et al.* [1922] have shown that an inverse ratio exists between the numbers of bacteria and protozoa in soil, bacteria being abundant when protozoa are few. When sufficient available food material is supplied to a mixed flora such as exists in soil or in a filter, interdependent variations in the numbers of organisms may occur without any alteration in the rate of oxidation of foodstuff. With a large excess of nutrient substance, however, biological variations might conceivably affect the rate of oxidation of the food substance.

The losses of water by evaporation in Exp. 2 were less than 1 % (Tables VI and X) and confirm the small loss recorded in Exp. 1. The figures for nitrogen given in Table VII show that in the filter receiving a solution with a C:N ratio of 8·4:1, 12·8 % of the nitrogen disappeared. With the larger ratio of 84:1 however a small gain of 0·057 g. or 5·7 % of the nitrogen supplied was recorded (Table XI). The difference between duplicate analyses of total nitrogen by the Kjeldahl method was of the order of 0·15 cc. $N/15$ acid with 250 cc. effluent. Assuming that this difference always operated in one direction, recording, say, a greater amount in the solutions than was actually present, then a gain of less

Table VI. *Filter A. C:N ratio 8·4:1.*
Volume balance sheet (Exp. 2).

Period days	Volume filtered cc.	Volume recovered as effluent cc.	Volume retained by filter *plus* vol. lost by evaporation cc.
0–7	12,748	12,608	140
8–14	12,568	12,435	133
15–21	13,062	12,998	64
22–28	22,927	22,544	383
29–35	25,382	25,153	229
36–42	25,067	24,773	294
43–51	25,004	24,303	701
52–58	16,399	16,436	−37
Total	153,157	151,250	1907

Liquid in filter = 667
Loss by evaporation = 1240 = 0·8 %

Table VII. *Filter A. C:N ratio 8·4:1.*
Nitrogen balance sheet (Exp. 2).

Period days	Nitrogen added as NH_4HCO_3 g.	Nitrogen recovered in effluent, g. Total N	NH_3-N	Organic N	Section No.	Nitrogen recovered in film, g. Total N	NH_3-N	Organic N
0–7	0·6304	0·3047	0·0145	0·2902	1	1·0135	0·1464	0·8671
8–14	0·6218	0·5054	0·3396	0·1658	2	0·5064	0·1344	0·3720
15–21	0·6499	0·4550	0·3397	0·1153	3	0·2639	0·1033	0·1606
22–28	1·1222	0·5629	0·4653	0·0976	4	0·1578	0·1062	0·0516
29–35	1·2577	0·5582	0·4745	0·0828	Total	1·9416	0·4903	1·4513
36–42	1·2387	0·5974	0·4350	0·1624				
43–51	1·2152	0·9361	0·5745	0·3616				
52–58	0·8218	0·7305	0·4035	0·3270				
Total	7·5577	4·6502	3·0475	1·6027				

Nitrogen recovered = 4·6502 + 1·9416 = 6·5918 g.
Nitrogen unaccounted for = 7·5577 − 6·5918 = 0·9659 g. = 12·8 %

than 0·041 g. nitrogen would not be significant. The increase with Filter B was 0·057 g., indicating some fixation of atmospheric nitrogen.

Christensen and Fulmer [1927] have shown that the nitrogen in yeast is not recovered quantitatively by the ordinary Kjeldahl method and that 15 % of the nitrogen may be lost unless precautions are first taken to break down the cyclic nitrogen compounds which are believed to be the source of the low results. In view of the losses of nitrogen in the experiments already described determinations of nitrogen in the film were made by the usual Kjeldahl method and by the modified Kjeldahl method of Christensen and Fulmer [1927] which involved preliminary oxidation of the material with hydrogen peroxide and sulphuric acid. The nitrogen in 100 cc. samples of a suspension of film from the first section of Filter A was found by evaporating the solution to dryness and carrying out the analysis by the two methods. The volumes of $N/10$ HCl neutralised in the Kjeldahl determinations were 15·2, 14·75, and 14·95 cc.; average 14·97: in the Christensen and Fulmer method the figures were 14·8, 15·1, and 14·9; average 14·93, showing that the nitrogen in the film is recovered as completely by the ordinary Kjeldahl method as by the peroxide modification.

The amounts of phosphorus and potassium recovered (Tables VIII, IX, XII and XIII) while greater in proportion than those in Exp. 1, were less than the

amounts supplied. The amounts of C, N, P_2O_5 and K_2O in the biological films of the two filters were:

Amount of C, N, P_2O_5 and K_2O in 100 g. of dry film.

	C:N 8·4:1	C:N 84:1
	g.	g.
C	46·2	46·2
N	7·0	3·5
P_2O_5	2·54	4·83
K_2O	1·18	3·38

Table VIII. *Filter A. C:N ratio 8·4:1. Potash balance sheet (Exp. 2).*

Period days	K_2O added in nutrient solution g.	K_2O recovered in effluent g.	Section No.	K_2O recovered in film g.
0–7	0·5071	0·2823	1	0·1501
8–14	0·5001	0·5429	2	0·0508
15–21	0·5198	0·4710	3	0·0367
22–28	0·9122	0·7253	4	0·0089
29–35	1·0101	1·0185	Total	0·2465
36–42	0·9976	0·9571		
43–51	0·9952	1·0452		
52–58	0·6525	0·5745		
Total	6·0946	5·6168		

K_2O recovered = 5·6168 + 0·2465 = 5·8633 g.
K_2O unaccounted for = 6·0946 − 5·8633 = 0·2313 g.

Table IX. *Filter A. C:N ratio 8·4:1. Phosphorus balance sheet (Exp. 2).*

Period days	P_2O_5 added in nutrient solution g.	P_2O_5 recovered in effluent g.	Section No.	P_2O_5 recovered in film g.
0–7	0·3824	0·2256	1	0·2424
8–14	0·3770	0·3362	2	0·1521
15–21	0·3919	0·2900	3	0·0787
22–28	0·6878	0·3526	4	0·0548
29–35	0·7815	0·3272	Total	0·5280
36–42	0·7502	0·3633		
43–51	0·7501	0·6230		
52–58	0·4920	0·4276		
Total	4·6129	2·9455		

P_2O_5 recovered = 2·9455 + 0·5280 = 3·4735 g.
P_2O_5 unaccounted for = 4·6129 − 3·4735 = 1·1394 g.

Table X. *Filter B. C:N ratio 84:1. Volume balance sheet (Exp. 2).*

Period days	Volume filtered cc.	Volume recovered as effluent cc.	Volume retained by filter *plus* vol. lost by evaporation cc.
0–7	12,927	12,775	152
8–14	13,028	12,852	176
15–21	12,968	12,796	172
22–28	21,978	21,562	416
29–35	25,311	25,172	138
36–42	25,443	25,351	92
43–51	24,153	23,885	268
52–58	21,775	21,688	87
59–65	24,245	23,895	350
66–70	17,445	17,425	20
Total	199,273	197,402	1871

Water in filter = 445
Loss by evaporation = 1871 − 445 = 1426 = 0·7 % of volume filtered

Exp. 3. In a large-scale experiment carried out at the beet-sugar factory at Colwick in order to test the value of inoculating a percolating filter with selected organisms, two adjacent filters were used. Each filter was 6 ft. deep and contained 2 cu. yd. of graded gravel. Effluent from the factory was treated on these filters, one of which was inoculated at intervals with certain selected organisms while the other filter, which was not inoculated, served as a control. The factory effluent differed from the liquids filtered in the laboratory experiments but for

Table XI. *Filter B. C:N ratio 84:1. Nitrogen balance sheet (Exp. 2).*

Period days	Nitrogen added as NH_4HCO_3 g.	Nitrogen recovered in effluent, g. Total N	NH_3-N	Organic N
0–7	0·0646	0·0525	0·0155	0·0370
8–14	0·0651	0·0230	0·0033	0·0197
15–21	0·0648	0·0292	0·0027	0·0265
22–28	0·1099	0·0356	0·0000	0·0356
29–35	0·1266	0·0602	0·0000	0·0602
36–42	0·1272	0·0492	0·0048	0·0444
43–51	0·1208	0·0537	0·0000	0·0537
52–58	0·1089	0·0711	0·0000	0·0711
59–65	0·1212	0·0991	0·0554	0·0437
66–70	0·0872	0·0770	0·0214	0·0556
Total	0·9963	0·5506	0·1031	0·4475

Section No.	Nitrogen recovered in film. g. Total N	NH_3-N	Organic N
1	0·3343	0·0288	0·3055
2	0·1064	0·0096	0·0968
3, 4	0·0620	0·0000	0·0620
Total	0·5027	0·0384	0·4643

Nitrogen recovered = 0·5506 + 0·5027 = 1·0533 g.
Gain in nitrogen = 1·0533 − 0·9963 = 0·0570 g. = 5·7 %

Table XII. *Filter B. C:N ratio 84:1. Potash balance sheet (Exp. 2).*

Period days	K_2O added in nutrient solution g.	K_2O recovered in effluent g.
0–7	0·5143	0·5000
8–14	0·5183	0·4619
15–21	0·5160	0·3936
22–28	0·8745	0·6581
29–35	1·0072	0·8420
36–42	1·0127	0·7452
43–51	0·9611	0·9204
52–58	0·8664	0·8193
59–65	0·9648	0·9350
66–70	0·6942	0·5255
Total	7·9295	6·8010

Section No.	K_2O recovered in film g.
1	0·2585
2	0·1402
3 and 4	0·0893
Total	0·4880

K_2O recovered = 6·8010 + 0·4880 = 7·2890 g.
K_2O unaccounted for = 7·9295 − 7·2890 = 0·6405 g.

the purpose of the present paper its chief interest lies in the fact that it contained nitrogen in organic combination and only a negligible amount of ammoniacal nitrogen. During the first 9 days of the experiment (Oct. 21st–29th, 1931), only one set of samples was collected and analysed. During the remaining period of the experiment, Oct. 30th, 1931 to Jan. 4th, 1932, representative samples of the crude liquor pumped to the supply tank for the filters and of the treated effluents were collected each day. The results of nitrogen determinations in these samples and in the biological film collected from the media of the filters at the end of the experiment are given in Table XIV. From the nitrogen balance sheet also given in Table XIV, it appears that the total amounts of

nitrogen recovered in the effluents and in the films were 21 to 24 % less than in the untreated liquid.

Table XIII. *Filter B. C:N ratio 84:1. Phosphorus balance sheet (Exp. 2).*

Period days	P_2O_5 added in nutrient solution g.	P_2O_5 recovered in effluent g.
0–7	0·3878	0·3371
8–14	0·3908	0·3830
15–21	0·3890	0·3268
22–28	0·6593	0·5435
29–35	0·7593	0·6131
36–42	0·7633	0·5948
43–51	0·7246	0·5545
52–58	0·6533	0·4841
59–65	0·7274	0·5372
66–70	0·5234	0·4372
Total	5·9782	4·8114

Section No.	P_2O_5 recovered in film g.
1	0·3535
2	0·1744
3 and 4	0·1124
Total	0·6403

P_2O_5 recovered = 4·8114 + 0·6403 = 5·4517 g.
P_2O_5 unaccounted for = 5·9782 − 5·4517 = 0·5265 g.

Table XIV. *Nitrogen balance sheet of large-scale percolating filters (Exp. 3).*

Date 1931	Volume crude liquor filtered gallons	Total N in crude liquor	Total N in effluent from inoculated filter	Total N in effluent from uninoculated filter	Total N supplied by crude liquor to each filter in lbs.	Total N in lbs. recovered in: Inoculated filter effluent	Total N in lbs. recovered in: Uninoculated filter effluent
		Parts per 100,000					
Oct. 21–29	329	4·850	3·472	3·774	0·1596	0·1142	0·1242
„ 30	50	5·476	1·490	1·490	0·0274	0·0075	0·0075
„ 31	50	5·376	1·030	1·030	0·0269	0·0052	0·0052
Nov. 1	30	4·603	0·784	0·784	0·0138	0·0023	0·0023
„ 2	50	6·260	1·378	1·288	0·0313	0·0069	0·0064
„ 3	100	4·839	1·568	1·523	0·0484	0·0157	0·0152
„ 4	100	4·245	1·187	1·299	0·0425	0·0119	0·0130
„ 5	100	3·158	1·255	1·277	0·0316	0·0126	0·0128
„ 6	100	3·069	1·143	1·120	0·0307	0·0114	0·0112
„ 7	200	3·147	0·829	0·974	0·0629	0·0166	0·0195
„ 8	200	3·584	0·986	1·030	0·0717	0·0197	0·0206
„ 9	200	2·878	1·064	1·098	0·0576	0·0213	0·0213
„ 10–14	1000	3·181	1·109	1·109	0·3181	0·1109	0·1109
„ 15–19	1100	2·890	1·154	1·288	0·3179	0·1269	0·1417
„ 20–23	1000	1·613	0·851	0·851	0·1613	0·0851	0·0851
„ 24–28	1250	1·915	0·918	0·918	0·2393	0·1147	0·1147
„ 29–Dec. 3	1250	2·117	1·019	0·907	0·2646	0·1274	0·1134
Dec. 4–8	1250	3·819	1·456	1·345	0·4773	0·1820	0·1681
„ 9–13	1250	2·800	1·512	1·501	0·3500	0·1890	0·1876
„ 14–18	1250	2·710	1·456 (?)	1·456	0·3387	0·1820	0·1820
„ 19–24	1500	2·128	1·546	1·725	0·3192	0·2319	0·2587
„ 30–Jan 4, 1932	900	3·342	1·997	3·062	0·3008	0·1797	0·2759
Total					3·6916	1·7749	1·8973

Summary	Inoculated filter		Uninoculated filter	
Total nitrogen added, lbs.		3·6916		3·6916
Nitrogen recovered: (*a*) in effluent	1·7749		1·8973	
(*b*) in film	1·0420	2·8169	1·0080	2·9053
Total N lost		0·8747 = 23·7 %		0·7863 = 21·3 %

The conditions of experiment with these large filters at the factory could not be so definitely controlled as with the smaller laboratory filters. At the factory the acidity of the crude liquor increased during the 24 hours for which it was stored. As the acidity increased solid matter was precipitated and only a portion of this passed on to the filter; part was lost whenever the supply tanks were cleaned out. It is probable therefore that the true losses of nitrogen were really less than the amounts indicated in Table XIV.

Discussion of results.

Loss of nitrogen in biological reactions has frequently been supposed [Waksman, 1931, p. 481] to result from the reduction of nitrites or nitrates to gaseous nitrogen. In order to determine the extent of this type of reduction Muntz and Lainé [1911] filtered through soil crude sewage to which nitrate had been added. In a previous paper [Jenkins, 1933, 2] the author gave the results of experiments in which solutions of sugar and nitrite, sugar and nitrate and sugar and ammonium chloride were treated in biological filters, and these results showed that large proportions of the nitrogen present in the original solutions were not recovered in the effluents. Barritt [1931, 2] has shown that the reaction between nitrate and amino-acid in a biological environment results in loss of elementary nitrogen from the nitrate, the amino-acid remaining intact above p_H 6·0. Another explanation of loss of nitrogen from soil which Waksman [1931, p. 484] considers possible is the interaction of ammonia and nitrous acid, with the production of free nitrogen. In a study of the biochemistry of water-logged soils Subrahmanyan [1917] concluded that organic nitrogenous compounds were decomposed in water-logged soils and that ammonia was split off; the losses of nitrogen which may occur in such soils are, according to this author, due to the volatilisation of ammonia and not to the liberation of the free element. Davy [1814] concluded that nitrogen was lost from manure by fermentation and suggested that the formation of ammonia was an intermediate stage of the reaction. The author indicated in a previous paper [Jenkins, 1933, 2] that considerable amounts of nitrogen were unaccounted for when nitrogen was supplied to a percolating filter as nitrite, nitrate or ammonia. It is a well established fact that nitrites and nitrates can supply oxygen to certain organisms provided anaerobic conditions are maintained. It is equally well established that when anaerobic conditions exist in a percolating filter nitrification does not take place. Again, in order that volatilisation of ammonia may occur, as was suggested by Subrahmanyan [1917], the p_H of the medium must be above 7·0. Now in the laboratory tests described in Exps. 1 and 2, the initial p_H of the nutrient solution was 6·0 and the p_H of the final effluent in the first experiment was 3·0 for more than half the experimental period. As was previously stated the reduction in p_H of the solution from 6·0 to 3·0 was caused by the liberation of mineral acid from ammonium chloride, owing to the uptake of ammonia from the ammonium chloride supplied: in the second experiment the source of nitrogen was ammonium bicarbonate and the filtrate had the same p_H as the nutrient solution, *viz.* 6·0. The filters in the second experiment never produced nitrite or nitrate, although they did not choke and become anaerobic. The formation of nitrite and nitrate evidently requires a prolonged time of passage through the filter or a time of contact with the medium which the tubular glass filling used for the experiments does not provide. Hence it seems that neither nitrification followed by subsequent liberation of free nitrogen, nor volatilisation of ammonia from the surface of the media accounts for the loss of nitrogen found in the experiments in which

a C:N ratio of 8·4:1 was maintained. A suggestion has been made by Waksman [1931, p. 484] that nitrogen gas is produced from organic compounds through biological agencies. The course of the decomposition according to this writer is first the formation of ammonia, which then undergoes biological oxidation to nitrogen either by oxygen or hydrogen peroxide. If there were any extensive ammonification of organic compounds in a percolating filter it is likely that some indication of its occurrence would be shown by the appearance of ammonia, particularly when conditions in the film did not favour ammonia oxidation. The losses of nitrogen in Exp. 3 amounted to an average of 22·5% for the two filters calculated on the total nitrogen supplied. The nitrogen in the crude liquor consisted mainly of protein though some betaine and other organic sources of nitrogen were no doubt present. Although the nutrient liquid fed to the filters contained traces of ammoniacal nitrogen, *i.e.* about 0·1 part N per 100,000, the concentration of nitrogen as ammonia in the effluents never exceeded, and was generally less than, this figure. Moreover, nitrite as measured by *meta*phenylenediamine was only found in traces on one day out of the 76 days of the experiment. Consideration of the results of this last experiment point to an appreciable loss of nitrogen from organic matter submitted to biological filtration. According to evidence put forward in this paper, the loss appears to be caused by the direct liberation of free nitrogen, without the intermediate formation of ammonia or nitrite or nitrate. This of course does not preclude the occurrence of such intermediate reactions within the cells of the organisms responsible for the liberation of nitrogen. The question therefore arises: Is there any evidence which might point to the existence of organisms in percolating filters or soils which are able to effect the breakdown of organic nitrogenous compounds directly to gaseous nitrogen? A search of the literature on this subject has revealed only one reference to an organism of this nature. Wood and Wilcox [1897] claimed to have isolated an organism able to liberate free nitrogen directly from organic matter. Their work has not as yet been confirmed. It has been shown in this paper that ammonium compounds undergo decomposition in percolating filters with loss of nitrogen, such loss being influenced largely by the C:N ratio of the solutions filtered. Losses of nitrogen from solutions containing ammonia were recorded when this ratio was 8·4:1 while with organic sources of nitrogen (Exp. 3) the C:N ratio was of the order of 20:1. If the ratio is increased to 84:1 the recovery of nitrogen is quantitative and there is also a slight but significant gain due possibly to the fixation of atmospheric nitrogen.

The quantitative relationship between the amount of carbohydrate oxidised and the nitrogen used in the oxidation (assumed to be the difference between the nitrogen supplied and the ammoniacal nitrogen recovered in the film and in the effluents) will be seen from Table XV to differ considerably in the two filters.

It is evident from these results that to obtain complete oxidation of sugar by biological filtration carbon and nitrogen must be present in solution in a ratio not exceeding 62:1, since these elements are removed in this ratio when a limited amount of nitrogen and excess of sugar are filtered. With a C:N ratio of 62:1 the most effective use is made of the nitrogen supplied, but in order (*a*) to ensure sufficient nitrogen to build up an efficient film, (*b*) to obtain complete decomposition of sugar and (*c*) to allow for losses of nitrogen washed out of the filter as organic matter, a smaller ratio than 62:1 is required. Although carbon and nitrogen were supplied in the ratio of 8·4:1 in Filter A these elements were removed in the ratio of 15·4:1 so that a large wastage of nitrogen occurred. It follows therefore that complete oxidation of sugar and most effective use of

nitrogen are obtained when the C:N ratio lies between 15·4:1 and 62:1: a ratio of the order of 30:1 would probably satisfy these conditions.

The quantities of nitrogen assimilated in the combustion of 100 g. of sugar in Filters A and B are given in column 8. These figures are of interest in the light of experiments made by Hutchinson and Richards [1921] in which it was observed that when straws were decomposed by micro-organisms a definite relationship existed between the amount of carbohydrate decomposed and the amount of nitrogen immobilised. Later the term "nitrogen factor" was introduced to define the quantity of added nitrogen immobilised in decomposing 100 g. of straw, and Richards and Norman [1931] showed that this factor varied considerably in plant materials of different origin, that of flax straw being 0·46 and of oat straw 0·8. If these experiments are to be compared with those obtained by the decomposition of straw in heaps, the differences between the two methods should be recognised. The nitrogen not immediately used in a percolating filter passes out of the filter and is lost whereas in straw decompositions nitrogen in the inorganic form is available for microbial requirements. Moreover, the carbohydrate decomposed in the experiments described in this paper was pure and in solution while Richards and Norman [1931] used mixtures of solid polysaccharides. It is of interest to note that whereas the nitrogen factor of a given plant material remains approximately the same whether sufficient or excess of nitrogen is supplied, with a carbohydrate solution undergoing biological filtration the factor varies according to the C:N ratio used.

The amounts of carbon oxidised and carbon fixed as film are given in Table XV. The figures show that the quantities of carbon recovered as film in Filters A and B for every 100 g. of carbon oxidised were 16 g. and 11·5 g. respectively.

Table XV. *Amount of sugar oxidised and nitrogen used, in g., in Filters A and B.*

	Col. 1 Sucrose added	Col. 2 Sucrose recovered in effluent	Col. 3 Sucrose oxidised Col. 1 – Col. 2	Col. 4 Nitrogen added	Col. 5 Nitrogen recovered as NH_3 in effluent	Col. 6 Nitrogen recovered as NH_3 in film
Filter A. C:N=8·4:1. Operated for 58 days	153	6	147	7·558	3·048	0·490
Filter B. C:N=84:1. Operated for 70 days	200	74	126	0·996	0·103	0·038

	Col. 7 Nitrogen used Col. 4 – Col. 5 – Col. 6	Col. 8 Ratio: $\frac{\text{Sucrose oxidised}}{\text{Nitrogen used}}$	Col. 9 Ratio: $\frac{\text{Carbon oxidised}}{\text{Nitrogen used}}$	Col. 10 Carbon oxidised	Col. 11 Carbon in film on filter
Filter A. C:N=8·4:1. Operated for 58 days	4·020	$\frac{100}{2·73}$	$\frac{15·4}{1}$	61·9	9·9
Filter B. C:N=84:1. Operated for 70 days	0·855	$\frac{100}{0·68}$	$\frac{62·1}{1}$	53·1	6·1

Summary.

1. A study has been made of the decomposition of sucrose in the presence of inorganic salts of nitrogen, phosphorus and potassium by the method of biological filtration. Glass percolating filters, allowing for the recovery of organic film, were used to draw up balance sheets for the volume of liquid filtered and

the amounts of nitrogen, phosphorus and potassium added. The recovery of the last two elements was not quantitative, possibly owing to inadequate methods of analysis. The losses of water due to evaporation were 1·8 % in one experiment which lasted for 108 days; losses of 0·8 % and 0·7 % were found in filters which were operated for 58 and 70 days respectively.

2. In two experiments the solutions filtered contained sugar and ammonium salts sufficient to provide C:N ratios of 8·4:1 and in the third the ratio was 84:1. The balance sheets for nitrogen showed that 14 and 13 % of the total nitrogen supplied was lost in the case of the first two filters while a slight gain, equal to 5·7 % was recorded with the third filter.

3. Two large-scale filters were fed for 76 days with beet-sugar factory effluents. These effluents contained sugar and organic acids derived from sugar, and nitrogen in the form of organic compounds. The losses of total nitrogen were 24 and 21 % of the amount supplied, but these figures include some nitrogen lost in cleaning out the tanks used for storing the factory effluents. The C:N ratio of the solutions filtered was approximately 20:1.

4. When filters were supplied with an ammonium salt as the source of nitrogen, neither nitrite nor nitrate was detected in the effluents. There is thus no evidence for assuming that where nitrogen was lost from the filters nitrite or nitrate occurred as an intermediate compound. When the source of nitrogen supplied to the filters was organic, neither ammonia nor oxidised compounds of nitrogen were found. Consequently it cannot be maintained that the losses of nitrogen from these filters arose from the formation of ammonia or the production of nitrite or nitrate and subsequent denitrification. It appears, therefore, that the liberation of nitrogen from ammonia and organic compounds of nitrogen may be carried out entirely within the cell of an organism.

5. For every 100 g. of carbon as sugar oxidised, the amounts of carbon fixed in the filters as film were 16 g. and 11·5 g. when the C:N ratios of the nutrient solutions were 8·4:1 and 84:1 respectively.

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XXXIX. THE RELATIVE VELOCITIES OF THE PHOTOCHEMICAL REACTIONS OF CAROTENE AND VITAMIN A WITH RADIATION OF WAVE-LENGTH 2650 Å.

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The transformation of carotene into vitamin A *in vivo* has been demonstrated by Moore [1929] and carotene is now regarded as pro-vitamin A; but attempts to bring about the same transformation *in vitro* have not met with success [Ahmad, 1931; Euler, 1931]. Recently two claims to have obtained vitamin A from carotene *in vitro* have been put forward. Olcott and McCann [1931] reported that they had observed the transformation in the presence of minced liver tissue, and Bowden and Snow [1932] obtained evidence which led them to believe that the same change had been induced by photochemical action. The inadequacy of the evidence brought forward by each of these groups of workers was strongly criticised by Moore and Woolf [1932], who laid down the criteria for the successful accomplishment of the production of vitamin A from carotene. In the absence of further confirmatory evidence and in view of the interest attached to the relationship of carotene and vitamin A, a study of the photochemistry of carotene and vitamin A was commenced by the writer, and the first results of the study are presented below.

Bowden and Snow [1932] irradiated a solution of carotene in *cyclo*hexane with monochromatic radiation of wave-length 2650 Å., since this radiation is conveniently obtained from the mercury lamp and corresponds to the absorption band of the carotene spectrum in the region 2700–2800 Å. [Dulière, Morton and Drummond, 1928]. In the words of these authors "After a few hours, a strong band appeared at a wave-length corresponding closely with that of the band at 3280 Å., which is already recognised as characteristic of vitamin A. Further, the solution gave a blue coloration with antimony trichloride in chloroform." On this evidence they based their claim to the production of vitamin A and hoped to substantiate it by means of biological tests with the irradiated carotene. The difficulty of performing this biological test was pointed out by Heilbron and Morton [1932] who also advanced reasons for doubting the claim made by Bowden and Snow.

Under these circumstances the following observations were made by the writer.

[1] Medical Research Scholar of the Worshipful Company of Grocers.

Experimental.

A monochromatic illuminator of wide aperture was employed for irradiating solutions of carotene and of vitamin A with radiation of accurately controlled wave-length. The source of radiation employed was a K.B.B. atmospheric type upright mercury arc which had been well "matured" and therefore could be expected to supply a reasonably constant density of radiation when fed with constant current in surroundings of uniform temperature [Ridyard, 1928]. The solutions to be irradiated were contained in quartz cells of capacities 1 to 5 cc. placed immediately behind the objective slit of the instrument. For each of the experiments to be described, the illuminator was adjusted so as to irradiate the reaction cell with radiation of wave-length 2650 Å., and the collimator and objective slits were set at widths which limited the total breadth of the band of radiation falling on the cell to 20 Å. At this setting, with the source used, the total energy of the radiation falling upon the reaction cell was approximately 1000 ergs per second. Throughout the work the solvent used was B.D.H. *cyclo*hexane "special pure for spectroscopy." The solutions during irradiation were stirred by the passage of a slow stream of bubbles of nitrogen, rigorously freed from traces of oxygen, which was passed in through a fine capillary reaching to the bottom of the cell. During the course of a long experiment some evaporation of the solvent was found to occur, and to prevent any change in concentration of the solution from this cause it was necessary to examine the reaction cell at regular intervals and maintain the level of the solution constant by the addition of drops of pure *cyclo*hexane. A number of experiments were performed, of which the following are typical.

Exp. 1. Irradiation of carotene. A solution of carotene in *cyclo*hexane was prepared of concentration such that the yellow colour in a 1 cm. cell was matched by 12 yellow units in the Lovibond tintometer; *i.e.* $Y = 12$[1]. When 5 cc. of this solution were evaporated *in vacuo* to dryness and the residue dissolved in 5 cc. of chloroform, 0·5 cc. of the chloroform solution with 2 cc. of antimony trichloride reagent gave a faint blue colour requiring 0·4 *B* to match it in the tintometer. 1 cc. of the *cyclo*hexane solution was placed in a quartz cell of capacity 1·5 cc. and irradiated for 7½ hours with radiation of wave-length 2650 Å. At the end of that time the yellow colour of the solution had faded to a value of $Y = 6$, *i.e.* the carotene concentration had fallen to 50 % of its initial value. When the solution was evaporated *in vacuo* and the residue dissolved in 1 cc. of chloroform, 0·5 cc. of the resultant chloroform solution with 2 cc. of antimony trichloride reagent gave a blue colour too faint and transient to match in the tintometer. The chromogenic power of the solutes had therefore decreased during the irradiation.

Exp. 2. The absorption spectrum of irradiated carotene. In order to examine the absorption spectrum of the irradiated carotene it was necessary to repeat Exp. 1, with a larger volume of solution. 3 cc. of the carotene solution, $Y = 12$, as used in Exp. 1 were irradiated under the same conditions as previously used, in a larger cell. At the end of 12 hours the yellow colour had fallen to $Y = 7$. The irradiated solution was transferred to the spectrophotometer and its absorption spectrum examined. The spectrum obtained was qualitatively identical

[1] All figures used in this paper for the physical constants and chromogenic powers with antimony trichloride of carotene and of vitamin A are taken from the table in which they have been collected from the original sources by Moore and Woolf [1932]. For the method of calculating the values of Y and B.U. see Moore [1930].

with that of the unirradiated carotene solution. No band could be detected in the region of 3280 Å., where the value found for $\log \frac{I_0}{I}$ was less than 0·1 for a beam traversing 2 cm. of the solution.

Exp. 3. Irradiation of vitamin A. A solution of B.D.H. vitamin A concentrate in *cyclo*hexane was prepared. The concentrate was a clear oil of high potency, giving 3000 B.U. per mg. and $E^{1\,\text{cm.}}_{1\,\text{mg. per c.c.}} = 102$ at 3280 Å. When 5 cc. of the solution were evaporated to dryness *in vacuo* and the residue dissolved in 5 cc. chloroform, 0·5 cc. of the chloroform solution with 2 cc. antimony trichloride reagent gave a blue colour requiring 4 *B* to match it in the tintometer. The solution therefore contained 20 B.U. of vitamin A per cc. 1 cc. of the *cyclo*hexane solution was irradiated for 7·5 hours under the same conditions as described for carotene in Exp. 1. At the end of the period, the solution was evaporated *in vacuo* and the residue dissolved in 1 cc. of chloroform. 0·5 cc. of the chloroform solution with 2 cc. antimony trichloride reagent gave a blue colour matched by 1 *B*. Thus the concentration of vitamin A had fallen to 5 B.U. per cc. or 25 % of the initial concentration.

Discussion.

Interpretation of the experimental observations. On the assumption that current views as to the molecular weight and extinction coefficient of vitamin A are substantially correct, the solutions of carotene and of vitamin A which were used in the above experiments were of the same order of molar concentration. Calculating from the values of *Y* and B.U. respectively, the carotene solution contained approximately 0·006 mg. per cc. and the vitamin A solution approximately 0·005 mg. per cc., so that the molarity of the vitamin A solution was about 30 % higher than that of the carotene solution.

From Exp. 1 it will be noted that a decrease in the chromogenic power of the carotene towards antimony trichloride accompanied the destruction of the carotene. From the known values for carotene and vitamin A, calculation shows however that an increase in chromogenic power is to be expected if 10 % or more of the carotene destroyed is transformed into vitamin A. It follows therefore that in Exp. 1 much less than 10 % of the carotene destroyed by irradiation was changed into vitamin A. A similar conclusion is reached from the results obtained in Exp. 2. During the 12 hours' irradiation the concentration of the carotene had decreased by an amount equivalent to 5 *Y*, which is approximately 0·0025 mg. per cc. If during this transformation of carotene each molecule of carotene had yielded one molecule of vitamin A, then the resultant concentration of the vitamin A formed must have been 0·0015 mg. per cc., causing a selective absorption at 3280 Å. large enough to give a value of 0·4 for $\log \frac{I_0}{I}$ in the 2 cm. tube which was used. The observed value of $\log \frac{I_0}{I}$ for the irradiated solution at this wave-length was less than 0·1, and the absorption was not selective, from which it is concluded certainly that less than 25 %, and probably very much less than 25 %, of the carotene destroyed was converted into vitamin A in the experiment described.

The photochemical transformation of carotene. The observations made in Exps. 1 and 2 record a destruction of carotene by radiation of wave-length 2650 Å., without the emergence of an absorption band in the region 3280 Å., and without any increase in the chromogenic power of the carotene and reaction products towards antimony trichloride. This suggests that little or none of the carotene

transformed in those experiments was converted into vitamin A. Such negative results alone would not however be sufficient to show that the product obtained photochemically from carotene contains no vitamin A. On the other hand, Exps. 1 and 3 give a comparison of the relative rates of destruction of carotene and of vitamin A by radiation of wave-length 2650 Å. Under standard conditions, in the same period, 75 % of vitamin A was destroyed and only 50 % of carotene, when the solutions irradiated were of the same order of concentration. From this it is concluded that vitamin A is more sensitive to the radiation than is carotene; and it follows that the end-product of the photochemical transformation of carotene cannot be vitamin A. The possibility is not of course excluded by these observations, that vitamin A may be an intermediate product in the photochemical transformation of carotene, but this is regarded as unlikely for the reason already given by other workers [Heilbron and Morton, 1932; Moore and Woolf, 1932], namely that if the experimental conditions are rigidly controlled, there can be no oxygen available for the conversion of the hydrocarbon carotene into the alcohol vitamin A.

The general application of photochemistry to vitamin studies. The observations of Bowden and Snow [1932] with regard to carotene could not be repeated, and it has been shown definitely that carotene cannot give rise to vitamin A when acted on by radiation of wave-length 2650 Å. As this is the only new observation of any significance brought forward by these authors in making their claims for the importance of photochemical methods in vitamin chemistry, it would seem that their claims are unjustified. The empirical application of irradiation methods has yielded invaluable results in the study of vitamin D, but the application of photochemical theory in the development of our knowledge of substances as complex as the vitamins is scarcely likely to be useful at present, because the photochemical behaviour of only the simplest compounds has so far been satisfactorily accounted for. The general assumptions made by Bowden and Snow must be regarded as unfounded until evidence is accumulated in support of them.

Apart from the well-authenticated formation of vitamin D from ergosterol, claims have now been made for the photochemical preparation *in vitro* of three vitamins. Rygh, Rygh and Laland [1932] claimed to have prepared vitamin C by a radiation method, but the claim was subsequently withdrawn by Rygh and Rygh [1932]. Bowden and Snow [1932] claimed to have accomplished a photochemical preparation of vitamin A, which is now shown to be unfounded, and Guha and Chakravorty [1932] have announced the formation of vitamin B_1 from adenine sulphate, a claim which cannot be appraised until their experimental work has been described.

Summary.

The observations of Bowden and Snow on the properties of irradiated carotene could not be repeated.

It has been shown that vitamin A is more rapidly destroyed than carotene by radiation of wave-length 2650 Å.

The possibility that vitamin A is the end-product of the photochemical reaction of carotene with this radiation is therefore definitely precluded.

I wish to record my great indebtedness to Prof. A. J. Allmand for permission to perform the irradiation experiments in the Department of Physical Chemistry, King's College, London, and also to Mr H. N. Ridyard and Mr H. C. Craggs for their constant advice and help in the use of the monochromatic illuminator, and for the measurement of the radiation density employed.

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XL. THE CHEMICAL NATURE OF VITAMIN C.

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(*Received January 13th, 1933.*)

The object of this paper is to show that the antiscorbutic activity of our "hexuronic acid" preparations is due to the acid itself and not to a contamination by some more potent substance. The authors think that they have demonstrated this and that the experiments presented give definite evidence for the identity of the acid and vitamin C. In accordance with the latest results of chemical analysis [Karrer *et al.*, 1933; Cox *et al.*, 1932] "hexuronic acid" will henceforth be called *ascorbic acid* [Szent-Györgyi and Haworth, 1933].

Preparation of ascorbic acid from paprika.

Progress in our work was dependent on the possibility of obtaining larger quantities of ascorbic acid. Unfortunately, adrenal glands, the only material heretofore suitable for large scale preparation, were not available in the necessary quantity. All our efforts to prepare ascorbic acid on the large scale from tomatoes, cabbages or oranges failed.

In a quest for more suitable material, we have found that local varieties of paprika (Hungarian red pepper, *Capsicum annuum*) showed a strikingly high reducing power. 1 cc. of the juice of the fresh and ripe fruit reduces in acid solution on the average 2·4 cc. of 0·01 *N* iodine or the equivalent quantity of dibromophenolindophenol. This would correspond to about 2 mg. of ascorbic acid if this substance were solely responsible for the reduction.

In animal experiments paprika juice shows a strong antiscorbutic activity. With 0·25 or 0·5 cc. of the juice, guinea-pigs were kept practically free from scurvy in a 53-day experiment (see Table II). Throughout this experiment the same juice was used, which was kept *in vacuo* at 0°. This brings out the fact, that paprika juice not only contains relatively large amounts of the antiscorbutic factor, but also contains it in a relatively stable condition, suitable for chemical work.

50 kg. of paprika, freed from the core, were minced in a meat grinder and 1750 g. of barium acetate in the form of a hot saturated solution were added. After standing for an hour the pulp was pressed out in a fruit press, 40 litres of juice being obtained. In absence of air this juice is stable for a period of 2 to 3 weeks. In the decanted clear liquid, 5 % of normal lead acetate is dissolved and the solution made slightly alkaline to bromothymol blue with ammonia. The precipitate is sharply separated on the centrifuge, suspended in very little water and 25 % sulphuric acid added till the fluid colours thymol blue slightly red. The lead sulphate is removed by centrifuging and 10 % of barium acetate is dissolved in the liquid. The inactive precipitate is removed on the centrifuge.

[1] Holder of an American-Hungarian Exchange Fellowship (1931–33) from the Institute of International Education, New York.

A sufficient quantity of lead acetate (5–15 %) is dissolved in the liquid, which ought to give no more precipitate at p_H 8·5 on the addition of excess of lead. The fluid is made alkaline to bromothymol blue, the precipitate sharply separated on the centrifuge, and treated as above. After the first lead treatment the volume is about 8000 cc., after the second about 1500–2000 cc. The total reducing power drops to one-half of the original value.

Ammonia is added till the fluid no longer affects thymol blue. The solution is distilled *in vacuo* with a low inside temperature (20–30°) to a syrupy consistency (200–300 cc.). About double the volume of methyl alcohol is added with constant stirring and the relatively inactive precipitate separated on a Büchner funnel. Then an equal volume of acetone is added and the fluid decanted from the resinous precipitate. The precipitate is extracted with a little methyl alcohol to which acetone is again added.

The combined extracts are concentrated to a syrupy consistency *in vacuo* to a volume of about 150 cc. 100 cc. of methyl alcohol are added and subsequently 500 cc. of acetone. The fluid is decanted from the precipitate which is extracted again with a small quantity of methyl alcohol. The second extract is treated with acetone and the fluids are combined. To this combined fluid, an equal volume of freshly distilled anhydrous ethyl ether is added. After decantation, the syrupy residue is extracted with methyl alcohol and the extract treated with acetone and ether. The combined fluids are concentrated *in vacuo* to a syrup. This syrup is transferred into a large flat-bottomed crystallisation dish and placed over sulphuric acid *in vacuo* till the next day. Granulated NaOH is placed in the same desiccator. On drying, most of the ascorbic acid separates in large well-formed crystals, which are freed from the resinous mother-liquor by washing with small amounts of methyl alcohol. The crystals, collected on a Büchner funnel, are washed with acetone and dried *in vacuo*. The entire preparation up to this stage takes on the average 3 days. The mother-liquor is treated once more with excess of acetone and ether as above and a second crystallisation effected.

The product consists of slightly yellow, well-formed crystals, melting at 187–189°; yield: 6·5 g. per 10 l. of juice, of which amount 5–6 g. separate on the first crystallisation. In animal experiments (see Table II) 0·5 mg. of the substance protects guinea-pigs against scurvy in a 65 day test period.

Recrystallisation. The crystals are dissolved with slight heating in a mixture of methyl alcohol (3 parts) and dioxan (2 parts), 5 cc. being used for every g. of the acid. The solution is distilled *in vacuo* to a small volume and left in the ice-box overnight. The crystals are washed with acetone and dried *in vacuo* over sulphuric acid. More crystals can be obtained from the mother-liquor on further concentration. The recrystallisation can be effected with very little loss.

The product consists of white, well-formed crystals, which melt sharply at 192°; $[\alpha]_D^{20°} = +24°$. Reduction equivalent weight is 88, so that the molecular weight is 176. The product thus corresponds to a pure preparation of ascorbic acid.

By this method about 450 g. of recrystallised ascorbic acid have been prepared in our laboratory.

Monoacetone-ascorbic acid.

If a crystalline preparation of a substance is found active in vitamin work, the question arises, whether the activity is due to the substance itself or to its contaminations. Even repeated recrystallisation of the substance does not

decide the question, since the contaminating substances might lend themselves to the formation of mixed crystals of constant composition. Definite evidence can be obtained by the preparation of a derivative of the substance in question. If the derivative is recrystallised and the original substance recovered and found active, little doubt is left about its identity with the active substance. There is very little chance that the contaminating substances should fit into the crystalline structure of the derivative in the same way as with the original substance.

In collaboration with Vargha [1932] we set out in search of a suitable derivative. Acetyl and benzoyl derivatives could not be crystallised. An almost ideally suitable derivative was found in the monoacetone derivative.

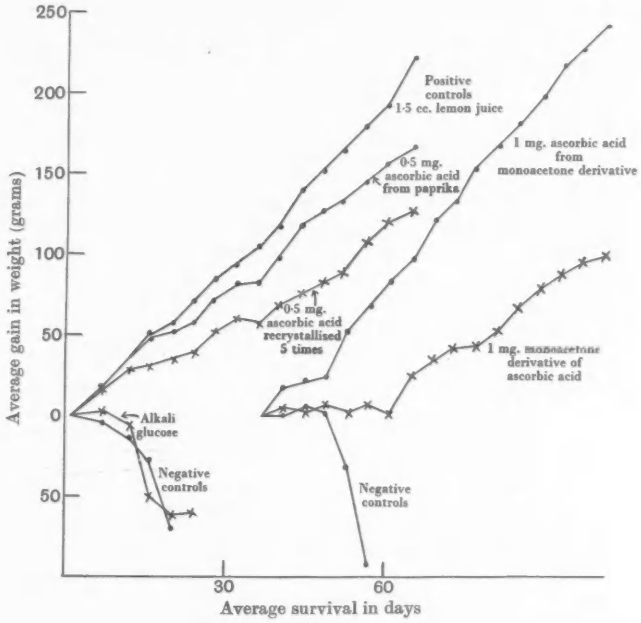

Fig. 1.

20 g. of ascorbic acid were shaken in 500 cc. of acetone in the presence of 50 g. of anhydrous copper sulphate for 24 hours. After filtration, the fluid was evaporated *in vacuo* to one-third of its volume. On addition of a double volume of light petroleum the monoacetone derivative of ascorbic acid crystallised out in large well-formed colourless prisms. These were dissolved in acetone and recrystallised by the addition of light petroleum; 70 % of the theoretical yield was obtained.

The acetone derivative is slowly decomposed in cold, readily in hot watery solution. If a fresh watery solution is quickly dried *in vacuo* at low temperature, the acetone derivative is recovered in crystals. If, however, the watery solution is heated for 15 mins. on the water-bath and then evaporated, ascorbic acid is obtained in crystalline form and can be identified by all its physical and chemical constants.

The acetone derivative was kept *in vacuo*, solutions being made immediately before feeding to the animals. The ascorbic acid recovered in crystalline form from the acetone derivative was administered in an analogous solution.

The results of this experiment can be seen in Fig. 1 and Table II. It will be noted that the ascorbic acid recovered from the acetone derivative is fully active, while the acetone derivative itself shows but a moderate activity which might be due to the acetone derivative itself or to its partial hydrolysis.

Recrystallisation experiment.

In order to obtain additional evidence for the activity of ascorbic acid, our original preparation, obtained from adrenal glands, was recrystallised five times using the original procedure, *i.e.* adding light petroleum to an anhydrous solution of ascorbic acid, using methyl alcohol as the primary solvent. After each crystallisation, the crystals were washed with acetone and dried[1].

The original M.P. was 184° and rose in the course of the recrystallisations to 187° showing that no entirely pure preparations can be obtained by this method. The crystals at the end of the fifth recrystallisation were well-formed but were slightly yellow.

For reasons outlined above, we do not attach too much value to such an experiment. The preparation obtained after five subsequent recrystallisations was found to be protective against scurvy in doses of 0·5 mg. in a 65 day test period (see Table II).

The ascorbic acid content of the adrenal gland.

If ascorbic acid is a vitamin we have to expect that animals liable to scurvy are unable to build up this substance. It has been shown in a previous communication by one of us (A. Sz.) that the adrenal cortex contains in the normal animal relatively large quantities of ascorbic acid. It seemed to be of interest to find out the behaviour of the ascorbic acid content of the gland on a vitamin C-free diet.

The ascorbic acid of the adrenal glands was estimated in the following manner. The adrenal glands of freshly killed guinea-pigs were weighed, then minced with sand in a small mortar. The pulp was suspended in 4 cc. of 1 % trichloroacetic acid and centrifuged. The fluid was titrated with dibromophenol-indophenol (0·1 %), which had been standardised against a known solution of ascorbic acid. The first permanent faint pink colour was regarded as the end-point, correction being made for the blank. Glutathione does not reduce the dye under the experimental conditions.

The results are given in Table I. As will be seen, the glands of animals which had been fed in addition to the basal diet on very liberal amounts of spinach for 10 days contain high amounts of the reducing substance. If the amount of spinach is restricted to about half a handful on alternate days, the values are much lower. The average value for the former group is 0·9 mg. ascorbic acid per g. of the adrenal gland, while the latter group shows an average of 0·2 mg.

Kept on a vitamin C-free diet for 20 days, the average value dropped to 0·03 mg. per g. This drop is already pronounced on the ninth day with an average value of 0·09 mg. per g.

[1] We express our gratitude to Prof. E. C. Kendall, Director of the Department of Chemistry of the Mayo Clinic at Rochester, Minn., for the supply of ascorbic acid from adrenal glands, which he was kind enough to send to us.

Table I.

	Weight of adrenals mg.	Final weight of animals g.	Ascorbic acid mg./g. gland
Very liberal diet of spinach	176	365	1·08
	490	730	0·8
	196	485	1·0
	234	305	0·6
	130	250	1·0
Restricted diet of spinach	265	490	0·2
	280	545	0·22
	290	480	0·2
	220	400	0·15
9 days on basal diet only	560	800	0·06
	195	240	0·08
	402	490	0·09
	174	320	0·15
	163	265	0·15
	627	330	0·08
	468	290	0·03
20 days on basal diet only	500	475	0·03
	580	645	0·03
	450	560	0·03
	473	525	0·03
	272	473	0·03
	273	160	0·03
1·5 cc. lemon juice	320	495	0·11
	228	415	0·12
	219	480	0·17
0·5 mg. ascorbic acid from paprika	440	515	0·09
	310	465	0·08
	327	545	0·10
1 mg. ascorbic acid from acetone derivative	290	585	0·11
	299	460	0·18
	231	495	0·15
	214	490	0·17

The positive controls which received 1·5 cc. lemon juice (minimum dose giving full protection against scurvy) show a fairly low value of 0·13 mg. per g. The animals receiving 0·5 mg. ascorbic acid from paprika juice (corresponding with 0·8 cc. lemon juice) had 0·09 mg. per g. The animals receiving daily 1 mg. ascorbic acid recovered from the acetone derivative showed 0·15 mg. per g. of gland.

We wish to emphasise the fact, that these results suggest that there is a wide limit between health and scurvy and that animals fed on restricted amounts of the vitamin, though not showing signs of scurvy, are greatly depleted of their vitamin store.

Similar results have been obtained lately by Moore and Ray [1932] who observed the disappearance of the silver reduction in the gland on vitamin-free diet.

Alkaline glucose solutions.

It is known that glucose treated with alkali at high temperatures gives rise to the formation of strongly reducing substances, with a similar reducing power to ascorbic acid.

10 % glucose solution was treated at 100° with *N* NaOH for 30 secs. The solution, after neutralisation, strongly reduced silver nitrate and iodine in acid

solution at room temperature. If the reduction had been due to the formation of ascorbic acid, 10 % of the glucose had been transformed into this substance.

1 cc. of the solution was fed daily to guinea-pigs. The animals, as seen in Fig. 1, lost weight in a similar way to the negative controls and died with symptoms of severe scurvy. Thus no ascorbic acid is formed on the treatment of glucose with alkali.

Experimental.

The general procedure used in testing the antiscorbutic activity of the different substances was that recommended by Sherman *et al.* [1922]. Instead of the customary 90 day period, a 65 day test period was chosen since definite results are also obtained by the shorter test period. The animals placed on the various tests varied in weight from 200 to 350 g., care being taken to ensure an equal distribution in regard to weight.

The solutions of the substances to be tested were administered daily by means of a graduated pipette.

No difficulty was encountered in feeding the paprika juice to the animals, who took it eagerly. The p_H of the juice as estimated by indicators was about 4.

Table II.

Fed daily	No. of animals	Average survival (65 days' test) days	Average scurvy score*	Average gain in weight g.
Basal diet only	3	19	13	−98
1·5 cc. lemon juice	3	65	0	222
0·125 cc. paprika juice	2	53†	7	−70
0·25 cc. ,,	3	53†	3	33
0·5 cc. ,,	3	53†	1	87
1 mg. monoacetone-ascorbic acid	4	65	1	100
1 mg. ascorbic acid from acetone derivative	5	65	0	243
0·5 mg. ascorbic acid from paprika	6	65	0	167
0·5 mg. ascorbic acid recryst. 5 times	4	65	1	129
Basal diet only	3	21	13	−83
1 cc. alkali glucose	6	19	17	−62

* Highest possible score is 24.
† Chloroformed owing to depletion of supply of paprika juice.

Summary.

It is shown that paprika (*Capsicum annuum*) contains ascorbic acid in relatively large quantities and under conditions which make its isolation fairly simple. The method of preparation is described.

The monoacetone derivative of ascorbic acid was prepared. It is shown that this substance is moderately active as an antiscorbutic. The ascorbic acid recovered from the monoacetone derivative is fully active. This is regarded as definite evidence concerning the identity of ascorbic acid and vitamin C.

It is shown that ascorbic acid (from adrenal glands) recrystallised five times retains its activity.

Ascorbic acid readily disappears from the adrenal glands of animals on a vitamin C-free diet.

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XLI. THE HYDROLYSIS OF HEXOSEDIPHOSPHORIC ESTER BY BONE PHOSPHATASE. A NEW FRUCTOSEMONOPHOSPHATE[1].

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It is remarkable that while at least three hexosemonophosphoric esters, those of glucose, fructose and mannose, are formed during the fermentation of sugar by yeast juice, only one diphosphoric ester, the hexosediphosphate of Harden and Young, has been discovered among the fermentation products. To this compound the constitution 1 : 6-diphosphofructofuranose has been ascribed [Morgan and Robison, 1928; Levene and Raymond, 1928; Morgan, 1929], and while this structure cannot be taken as definitely established it is supported by such strong evidence that it may be accepted as most probably correct.

The presence of a phosphoric acid group in position 1 was deduced by Young [1911] from the fact that the ester yielded a phenylhydrazone containing two phosphoric acid groups but lost one of these groups on forming an osazone. (The bare possibility must be recognised that a phosphate group in some other position might be so readily hydrolysed as to be split off during the reaction.)

The conclusion that the second group is in position 6 was based on evidence, obtained by methylation and dephosphorylation, that the ester is a furanose derivative containing the unstable 1 : 4 oxygen bridge. It is further supported by the identity of the osazone with that of glucosemonophosphoric ester to which the structure of glucose-6-phosphate has been assigned [Robison and King, 1931; Levene and Raymond, 1931].

When hexosediphosphate is submitted to hydrolysis by acids the phosphoric acid group in position 1 is removed much more rapidly than that in position 6. By this means Neuberg [1918] obtained a fructosemonophosphate (Neuberg ester) which yields the same osazone as that obtained from the diphosphoric ester, and which is, therefore, most probably 6-phosphofructofuranose. Like the parent fructosediphosphate this ester is very slightly dextrorotatory. More recently the same ester has been isolated from the hexosemonophosphate of fermentation, of which it probably represents the principal ketose component [Robison and King, 1931; Robison, 1932].

On removal of both phosphoric acid groups from hexosediphosphate by prolonged boiling of the aqueous solution of the free acid, Young [1909] obtained a laevorotatory syrup containing fructose; but he observed that the rotation of the crude syrup was very much lower than that of a solution of pure fructose of similar reducing power. He suggested that this might be accounted for by the presence of glucose or mannose but was not able to obtain experimental confirmation of this. From similar hydrolysis products pure crystalline fructose was later isolated by Neuberg, Färber, Levite and Schwenk [1917].

Neuberg and Leibowitz [1927] have investigated the products of the partial hydrolysis of fructosediphosphate by phosphatases of animal and plant origin.

[1] This paper was communicated to the Biochemical Society on October 21st, 1932, and an abstract was published in *Chemistry and Industry*, 1932, **51**, 913.

The enzyme preparations were allowed to act on magnesium fructosediphosphate in aqueous solution at 37° for periods up to 86 hours, the amount of hydrolysis representing from 20 % up to about 50 % of the total combined phosphorus. With the phosphatase of *Aspergillus oryzae* they obtained a hexosemonophosphate similar to the Neuberg ester in specific rotation and other characters. It may be noted, however, that the iodimetric reducing power (Auerbach-Bodländer) of their product was considerably higher than the value which they give for the Neuberg ester, which suggests that the product contained a higher proportion of aldose esters.

With fresh yeast in presence of chloroform and toluene the hydrolysis yielded a product intermediate in character between the Robison and Neuberg esters, while if the hydrolysis was effected with yeast activated with arsenate the properties of the monophosphate closely approached those of the Neuberg ester. The phosphatase of horse kidney on the other hand gave a hexosemonophosphate very similar to the Robison ester of fermentation, which is now known to be a mixture containing esters of glucose, mannose and fructose, the aldose esters preponderating.

Martland and Robison [1929] studied the hydrolysis of hexosediphosphate by bone phosphatase at 40°, and found that the specific rotation of the sugar produced, calculated on its reducing value (Hagedorn and Jensen), was in all cases much lower than that of fructose. This result was obtained whether the hydrolysis was carried out at p_H 7·0 or at p_H 8·8 and whether the reaction was completed in a few hours or in several days.

The results which have been enumerated provide evidence that during the removal of phosphoric acid groups from fructosediphosphate intramolecular change in the hexose portion of the molecule may, under certain conditions, be facilitated. This conclusion is the more interesting by reason of the analogous changes which take place during esterification of the sugars in alcoholic fermentation.

Experiments described in the present communication were planned with the object of gaining further knowledge of the changes taking place when fructosediphosphate is acted on by bone phosphatase, under conditions which would not be likely to cause fructose in the free state to undergo intramolecular transformation. From indications of previous experiments it was further hoped that this phosphatase might be found to hydrolyse the phosphoric acid group in position 6 as rapidly as that in position 1 and that the hitherto unknown ester, fructose-1-phosphate, might in this way be obtained. The methods employed for the isolation of the hydrolysis products were therefore chosen so as to minimise the risk of decomposition of this ester, if, as one might expect, it should prove to be unstable in acid or in hot aqueous solution.

The fructosediphosphate used for these experiments was prepared by the fermentation of glucose and fructose with dried yeast and was purified by precipitation of the barium salt from its solution on heating the latter to 70°, as recommended by Robison and Morgan [1930]. The preparations were examined by the analytical methods described in the same paper and the results were in satisfactory agreement with those given for barium hexosediphosphate, except that some of the samples contained a very small percentage of inorganic phosphate. The diphosphate used in the last experiment was, however, purified by a different method involving repeated precipitation of the acid salt from its aqueous solution by alcohol[1].

[1] (A full description of the method employed and of the results obtained in this fractionation of the sparingly soluble barium salts will shortly be published in collaboration with Dr M. G. Macfarlane. R. R.)

Purification of the bone phosphatase.

The phosphatase was obtained from the bones of young rabbits and was purified by precipitation from the filtered extract with alcohol and ether [Martland and Robison, 1929]. Since these preparations were found to possess a slight reducing power by the Hagedorn and Jensen method and a considerable iodimetric value, it was recognised that a further degree of purification would be advantageous if it could be effected without serious loss of enzyme activity or stability. With this object in view a large number of experiments were carried out in extension of those previously reported by Martland and Robison. The results, though valuable as a basis of further investigation, did not, at the time, provide us with any method of purification more reliable and satisfactory than that of alcohol-ether precipitation, which was therefore adopted for all phosphatase preparations used in the hydrolysis experiments. Precautions were, however, taken to minimise the risk of errors through contamination of the hydrolysis products with substances derived from the enzyme material.

The chief results of our experiments on the purification of the phosphatase are briefly summarised below.

Extraction of the alcohol-ether precipitate with aqueous alcohol. It was found that the phosphatase could be completely extracted from the alcohol-ether precipitates by 25 % alcohol (or acetone). With 50 % alcohol, extraction was sometimes complete but was more frequently accompanied by considerable diminution of the total activity. Precipitation of the enzyme from these extracts by the addition of alcohol and ether involved further loss of activity. Evaporation of the 50 % alcohol extracts to dryness in evacuated desiccators gave better results. It was important that the extract should be neutral or slightly alkaline in reaction. In one experiment 1·00 g. of an alcohol-ether precipitate, having a total activity of 320 units, was extracted with 50 cc. of 50 % alcohol; the activity of the extract did not increase after the first test, which was carried out after 1½ hrs. The extract, on evaporation to dryness, gave 134 mg. solid residue, having a total activity of 239 units. The ratio of activity to dry weight (A/W) had thus been increased from 0·32 to 1·7 with a loss of only 25 % of the total activity. Other results obtained by this method were, however, less satisfactory. The degree of extraction appears to vary with the character of the alcohol-ether precipitate; but the method remains one of the most promising for future exploration.

Extraction with 60 % and 70 % alcohol was very incomplete, while the enzyme was practically insoluble in concentrations of alcohol higher than 80 % (by volume). The enzyme could not be extracted from macerated bones by 50 % alcohol.

Table I. *Analyses of preparations of bone phosphatase.*

	Inorganic phosphate P %	Reducing power calculated as glucose		N % (micro-Kjeldahl)
		H. and J. %	I %	
Alcohol-ether precipitate (C)	—	8	24	—
Residue from 50 % alcohol extract of above	1·3	13 (27)	44 (48)	11·1
Residue from 50 % alcohol extract of another preparation	0·9	8 (15)	37	9·7
Alcohol-ether precipitate (D)	—	6	36	—
„ „ (E)	—	7	33	—

Analytical results obtained for some of these phosphatase preparations are shown in Table I. The figures in brackets represent reducing power after hydrolysis with 0·02 *N* HCl at 100° for short periods. The latter values were not increased by heating for longer periods with more concentrated acid.

Adsorption with norite and kaolin. Treatment of the filtered bone extracts with norite at different p_H values resulted in the removal of a little colouring matter; but no significant adsorption of phosphatase or of reducing material occurred, and no increase in the value of A/W was found. Kaolin had still less effect.

Precipitation by lead or mercuric acetate. Attempts to separate the phosphatase from the contaminating material by fractional precipitation with normal or basic lead acetate, or mercuric acetate, failed. The enzyme was not inactivated by lead salts but was destroyed when the solution or precipitates were treated with hydrogen sulphide.

Precipitation by alcohol at p_H *6.* It was found possible to precipitate the phosphatase from the filtered, aqueous extracts of bones by the addition of an equal volume of alcohol followed by adjustment of the p_H to 6·0–6·1, by cautious addition of 0·1 *N* HCl, the solution being cooled to 0°. The precipitate was removed by filtration or centrifugation, washed with absolute alcohol and dried *in vacuo*, or at once dispersed in boiled distilled water. In one experiment the value of A/W was increased about fivefold while some 60 % of the reducing substances were removed. This method, though less expensive than precipitation with alcohol-ether, was also less reliable, in that loss of activity was very apt to occur.

In the above experiments it was realised that partial loss of activity might arise from reduction in the magnesium content of the products [Erdtman, 1928; Jenner and Kay, 1931] and many of the tests were therefore carried out with and without the addition of magnesium chloride in 0·01 *M* concentration. The activity was in all cases raised by such addition.

Hydrolysis of hexosediphosphate.

Nature of the sugars produced by removal of both phosphate groups.

In Table II are summarised the results of 9 experiments on the hydrolysis of fructosediphosphate by 5 separate preparations of bone phosphatase. In order to lessen the risk of the spontaneous transformation of fructose into glucose and mannose the hydrolyses were carried out at p_H 7·0 and at room temperature or 25° instead of at 40° as in the experiments of Martland and Robison. Under these conditions the rate of hydrolysis was relatively slow but the enzyme suffered no serious inactivation even during periods of several weeks. The proportion of phosphatase to ester was varied within wide limits, being over 20 times as great in Exps. 1 and 5 as in Exps. 6, 7 and 9; the duration of the hydrolyses varied from 19 hours to 44 days.

Exps. 1–3 were carried out as follows. A solution (17–20 cc.) of potassium fructosediphosphate, prepared from the purified barium salt, was treated with the stated amount of phosphatase together with a few drops of chloroform; the p_H was adjusted to 7·0 and the hydrolysis allowed to proceed at room temperature, 0·2 cc. being removed from time to time for the determination of inorganic phosphate. The p_H rose gradually as the reaction proceeded but was kept within the limits 7·0–7·1 (rarely 7·2). When the hydrolysis was completed the remaining solution was treated with 4 times its volume of absolute alcohol, heated to boiling, filtered, and the residue washed with 80 % alcohol. The solution was evaporated

in a desiccator and the syrup extracted with boiling 90 % alcohol, the extract again evaporated to dryness, and the residue dissolved in 20 cc. H_2O for analysis.

In Exps. 4–8 the method of procedure was slightly modified. The hydrolysis was carried out at 25° and the reducing power (H. and J. and I) as well as the inorganic phosphate were estimated from time to time. The residual solution was directly evaporated to dryness in an evacuated desiccator and the residue repeatedly extracted with boiling 85 % alcohol, the filtered extract again evaporated to dryness and the syrupy residue examined as before. The total amount of sugar recovered as determined by the H. and J. reducing power is given in mg. and also as a percentage of the hexose equivalent to the phosphate set free, allowance being made for the amounts removed for analyses. The percentage recovery was not very good in the early experiments but reached 90 % and 95 % in numbers 7 and 8. Further quantities of reducing sugar were found on extracting the protein and phosphate residues with water, but these amounts have not been included in the table. Aldoses were determined iodimetrically [Macleod and Robison, 1929] while fructose was estimated by the Selivanoff reaction. No great accuracy can be claimed for these Selivanoff determinations but they form a useful check on the aldose values which might be vitiated by the presence of other substances capable of reacting with hypoiodite. In the majority of the experiments the sum of these two values agrees fairly well with the amount of total hexose (H. and J.). The specific rotation, calculated on the H. and J. value, varies from $-38°$ in Exp. 3 to $-93°$ in Exp. 7, the values corresponding roughly with the proportion of fructose found in the product. The proportion of aldose formed during the hydrolysis appears to vary with the enzyme preparation employed; but no conclusions can at present be drawn as to the cause of these variations. Certain of these syrupy products were treated with phenylhydrazine but the sparingly soluble hydrazone of mannose could not be obtained. They yielded, however, osazones apparently identical with glucosazone.

Table II. *Analyses of sugars produced on hydrolysis of hexosediphosphate with bone phosphatase.*

Exp	Duration days	Phosphatase prep.	Phosphatase weight mg.	Ester mg. P	Hydrolysis %	Sugar recovered (H. and J.) as % of calculated hexose	Reducing power as glucose: H. and J. mg.	Reducing power as glucose: I mg.	Fructose Selivanoff mg.	$[\alpha]_{5461}$ calc. on H. and J.
1	4·8	A	500	94	100	54	142	84	87	−48°
2	20	A	200	92	100	63	157	66	98	−45°
3	6·7	A	200	90	99	63	158	70	85	−38°
Controls	9	A	200	—	—	85	—	—	—	−102°
	9	A	None	—	—	84	—	—	—	−102°
4	29	A	50	94	97	71	137	34	104	−72°
5	0·8	B	200	32	94	75	64	27	47	−63°
6	11	C	100	430	50	—	111	36	100	−78°
	23	—	—	—	99	60	291	36	249	−87°
7	17	D	100	420	52	—	247	60	202	−78°
	42	—	—	—	94	90	190	25	—	−93°
8	4	D	140	95	96	95	127	26	114	−90°
9	44	E	400	1660	51	—	715 (+ 477)	256	448	−73°

Fructose has $[\alpha]_{5461} = -110·8°$.

Exp. 6. Total volume, 100 cc.; 40 cc. removed and examined after 11 days, 40 cc. after 23 days.
Exp. 7. Total volume, 100 cc.; 70 cc. removed and examined after 17 days, 18 cc. after 42 days.
Exp. 9. Total volume, 400 cc.; hydrolysis stopped and whole of solution examined after 44 days.

As already stated the phosphatase preparations possessed some reducing power, *e.g.* preparation *D* gave values of 6 %, H. and J., and 36 %, iodimetric,

as estimated on weighed portions of the solid, both values being calculated as "glucose." This reducing material was, however, almost insoluble in 85 % or 90 % alcohol, and could not account for the very low specific rotations of the recovered sugar. Control experiments were also carried out in which solutions containing equivalent amounts of fructose and inorganic phosphate, with and without addition of phosphatase, were allowed to remain at room temperature or 25° and were treated in a similar manner to the hydrolysis solutions. The results of two such control tests are given under Exp. 3. It will be seen that the specific rotation of the recovered sugar (− 102°) was somewhat lower than that of fructose, but was the same for the solution to which 200 mg. phosphatase had been added as for that containing fructose and phosphate only.

The determinations of reducing power, which were carried out during the course of the hydrolysis in Exps. 4–8, gave the following results. The H. and J. value increased steadily in all experiments as hydrolysis proceeded, the final value representing between 90 % and 100 % of the calculated amount of hexose. The iodimetric values showed much smaller and less regular increases; in Exp. 4 the reducing power increased by about 18 % of the total hexose within the first 6 days, thereafter remaining constant; while in Exp. 7 the total increase did not exceed 5–6 %, no significant change being observed after the first 3 days. In a subsequent experiment (No. 9), however, the changes were of greater magnitude and were more easily followed.

Nature of the hexosemonophosphates produced by partial hydrolysis of hexosediphosphate.

In Exps. 6 and 7 portions of the solutions (40 cc. and 70 cc. respectively) were removed for examination after 50 % of the total phosphate had been set free. In each case considerable amounts of free sugar giving $[\alpha]_{5461} - 78°$, were recovered, together with inorganic phosphate, unchanged hexosediphosphate and a small amount of hexosemonophosphate; the last-named was separated in the form of the readily soluble barium salt and purified by repeated solution in water and 10 % alcohol, and precipitation with alcohol. The weight of purified salt obtained from Exp. 6 was 88 mg. and from Exp. 7, 182 mg. Analyses (see Table III) indicated that they consisted largely of a fructosemonophosphate but probably contained some proportion (20–30 %) of aldose esters. Both salts were, however, laevorotatory, $[\alpha]_{5461} - 12°$, differing in this respect from any of the known hexosemonophosphates.

A further hydrolysis experiment (9) was therefore carried out on a larger quantity of fructosediphosphate, which had been carefully purified by repeated precipitation of its acid barium salt. The analysis of the neutral barium salt of this preparation is shown in Table III. Inorganic phosphate was absent. It is not to be inferred from the iodimetric value of 1·5 % that aldose esters were present, since the fructose ester might well be oxidised to a slight extent.

Method. A solution of the potassium salt (400 cc.), containing 1660 mg. P, was adjusted to p_H 6·9–7·0 and treated with 400 mg. of a bone phosphatase preparation. The solution was kept in a stoppered flask at 25° in presence of chloroform. 0·5 cc. was removed at intervals of 2 or 3 days and diluted to 25 cc. for the estimation of total and inorganic phosphate and reducing power. Hydrolysis proceeded regularly and reached 51 % in 44 days. The H. and J. reducing power increased during the same period from 41·5 % to 73·5 % of the total hexose equivalent. The iodimetric reducing value also increased gradually, as hydrolysis proceeded, from 5 % to 23 % of the total hexose. The p_H did not rise above 7·0.

Controls. Solutions containing (*a*) fructose + inorganic phosphate and (*b*) fructose + inorganic phosphate + phosphatase were kept at 25° and p_H 7·0 in presence of chloroform during 97 days and were examined at intervals. No decrease in the amount of inorganic phosphate was observed in either solution so that if synthesis of phosphoric ester occurred the amount cannot have exceeded 1 % of the total P. The H. and J. reducing power decreased in both solutions by about 6 % in 43 days, thereafter remaining nearly stationary; the iodimetric values increased from 1 % to 2 % (of the total hexose) in solution (*a*) and from 1 % to 3 % in solution (*b*), these changes taking place for the most part within the first few days. It is therefore clear that transformation of fructose into glucose or mannose does not occur to any considerable extent under these conditions. The loss of reducing sugar may have been due to oxidation by atmospheric oxygen.

Isolation of the products of hydrolysis. At the end of 42 days the remainder of the solution (370 cc.) was treated with basic lead acetate, the precipitate filtered, washed with water, and decomposed with the minimum excess of sulphuric acid, the solution being cooled in ice. The lead sulphate was removed by centrifuging and washed 4 times with water at 0°. The acid solution was at once brought to p_H 8·0 with cold baryta solution, and filtered. The filtrate and washings were treated with 3 times their volume of alcohol, and the precipitate was filtered off and purified by repeated solution in 10 % alcohol, and reprecipitation with alcohol.

Sugar. The filtrate from the basic lead precipitate contained 477 mg. of reducing sugar but the values found for this sugar are not shown as there is reason to believe it may have undergone some change during its isolation[1]. The greater part of the sugar formed in the hydrolysis was however found in the alcoholic filtrate from the barium salt. This was isolated and the analytical values are shown in Table II, Exp. 9. The sum of the amounts of aldose and fructose is in good agreement with the total hexose (H. and J.) but a mixture of glucose and fructose in these proportions should have $[\alpha]_{5461} - 48°$, whereas the value found was $- 73°$. The possibility that mannose might be present could not be confirmed as attempts to obtain the phenylhydrazone led to inconclusive results.

Hexosemonophosphates. Analyses of the sparingly soluble barium salt (8·85 g.) indicated that it consisted of inorganic phosphate and of unchanged hexosediphosphate (300 mg. organic P). The readily soluble barium salt (3·36 g.) was laevorotatory ($[\alpha]_{5461} - 10·1°$) and in this and other respects was very similar to the monophosphates isolated from Exps. 6 and 7. A small fraction (0·4 g.), obtained in the purification of the monophosphate, was of intermediate character.

The analytical results obtained for the hexosemonophosphate are given in Table III. Judged by the Selivanoff and iodimetric values, the product from this experiment appeared to contain some 30 % of aldosemonophosphates and 70 % of fructosemonophosphates.

Hydrolysis by N HCl at 100°. The composition of this salt was further studied by investigating the rate of hydrolysis in *N* HCl at 100°, the method of Lohmann

[1] The examination of this solution led to an interesting finding, namely, that fructose when dissolved in a concentrated solution of basic lead acetate (B.D.H. 25 % $Pb_2O(C_2H_3O_2)_2$) is dextrorotatory; 1 g. fructose in 30 % of this solution gave $[\alpha]_{5461} + 32°$; this value fell to $+26°$ in 1 hr. and to $+4°$ in 2 days at room temperature. On precipitating the lead with sulphuric acid the solutions became laevorotatory but this laevorotation also decreased the longer the fructose was allowed to remain in the basic lead acetate solution, indicating that the fructose had been permanently changed. 1 g. glucose in 30 cc. of this basic lead acetate solution gave $[\alpha]_{5461} + 51°$.

Table III. *Analyses of barium hexosemonophosphates obtained by partial hydrolysis of hexosediphosphate with bone phosphatase.*

Analyses of the three hexosemonophosphates obtained from the products of fermentation are given for comparison.

No. of salt	Source of the barium salt	P %	Fructose (Seli-vanoff) %	Reducing power as glucose (%) H. and J.*	Reducing power as glucose (%) Iodine	Aldose-mono-phos-phate %	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5461}^{20°}$ of free acid
1	Glucosemonophosphate	7·86	0·5	35·5	45·7	100	+20·6°	+41·4°
2	Mannosemonophosphate	7·82	2	36·9	27·0	59	+ 3·6°	+15·1°
3	Fructosemonophosphate	7·73	22	34·9	1·6	3	+ 2·3°	—
4	Hydrolysis 7; hexosemonophosphate	7·37	17	27·9	7·3	15	−11·8°	—
5	Hydrolysis 9; hexosemonophosphate	7·65	15	31·3	14·2	31	−10·2°	−17·3°
6	No. 5, after removal of aldose esters by oxidation with bromine; fraction (*b*) 20–70 % alcohol	7·85	24	25·9	1·5	3	−25·8°	—
7	No. 5. Residue after 43 % hydrolysis by 0·1 *N* HCl	7·27	11	31·0	23·4	51	+ 4·1°	
8	No. 5. Residue after 66 % hydrolysis by *N* HCl	7·51	2·6	33·4	38·2	84	+15·8°	—
9	Calculated values for new fructosemono-phosphate	7·85	25	23·0	1·5	—	−35°	—
10	Hexosediphosphate used for hydrolysis 9	10·04	9	12·0	1·5	5	+ 2·0° (in acid solution)	—

* Determined with addition of 0·5 cc. of 0·5 *N* NaOH.

[1928]. The hydrolysis of the hexosediphosphate, from which this monophosphate was obtained, was similarly investigated for comparison with Lohmann's values. Details of the method have already been given [Robison, 1932].

The initial rate of hydrolysis of the new monophosphate exceeded the high initial rate for hexosediphosphate, and this gave support to the view that a large proportion of the product consisted of a fructosemonophosphate in which the phosphoric acid group occupied position 1. Since position 6 would then be free, such a compound would probably possess the 1 : 5-oxygen bridge, and thus, as a derivative of normal fructose, might well be laevorotatory. Making use of the values of k previously found for the pure monophosphoric esters [Robison, 1932] it was calculated from the hydrolysis curve of the new product that this probably contained glucose- (or mannose-) monophosphate, Neuberg fructosemono-phosphate, and the second fructosemonophosphate in the proportions 28 : 20 : 52.

Removal of the aldosemonophosphates by oxidation with bromine. 1 g. of the barium salt, dissolved in 15 cc. H_2O, was treated at room temperature with 0·2 cc. bromine and 3 cc. of a cold, saturated solution of baryta; a further 6 cc. of the baryta solution was added gradually during 70 minutes; the solution was then made faintly acid with HCl and aerated to remove the bromine. After adjustment of the p_H to 8·4 with baryta, the solution was filtered and poured into 3 times its volume of alcohol. The precipitate was extracted with 10 cc. of water, the solution filtered from the sparingly soluble phosphohexonates and poured into alcohol. This purification was repeated twice more with substitution of 10 % alcohol for the water. The last traces of phosphohexonate were removed by fractional precipitation of the salt from aqueous solution with alcohol (*a*) to 20 %, (*b*) 20 % to 70 %. The analysis of fraction (*b*) is given in Table III. From the very low iodimetric value (1·5 %) it was concluded that aldose esters could only be present in negligible proportion, while the Selivanoff value (24 %) was even higher than that given by the Neuberg fructosemonophosphate. The H.

and J. reducing power was, however, decidedly lower than that of the three known monophosphates, which would seem to be reasonable if the phosphate group of the new ester is in position 1. The value of $[\alpha]_{5461}$ had been raised to $-25{\cdot}8°$.

This purified salt (*b*) was used for the investigation of the hydrolysis by *N* HCl at 100°. The initial rate was still higher than before, 44 % of the phosphate being set free in 5 minutes and 73 % in 15 minutes, but the calculated values of $k\left(\frac{1}{t_1-t_2}\log\frac{a-x_1}{a-x_2}\right)$ fell off rapidly from 50×10^{-3} to 4×10^{-3}. The amounts of hydrolysis agree closely with those calculated for a mixture containing 75 % of an ester having $k = 75\times10^{-3}$ and 25 % of the Neuberg ester having $k = 4{\cdot}2\times10^{-3}$ [Robison, 1932]. This conclusion is in good accordance with that deduced for the composition of the hexosemonophosphate previous to oxidation with bromine.

Table IV. *Hydrolysis of hexosemonophosphates obtained from hexosediphosphate in* N *HCl at 100°.*

	A Before oxidation (0·285 mg. P per cc.) Hydrolysis %		B After oxidation (0·283 mg. P per cc.) Hydrolysis %	
Time min.	Found	Calculated*	Found	Calculated†
5	31·4	31·1	44·0	44·6
10	44·2	44·6	64·1	64·0
15	50·4	51·0	72·8	72·8
25	55·1	55·0	79·1	79·4
40	58·5	59·0	81·7	82·9
60	61·0	61·7	84·7	86·0
90	64·8	64·9	—	—
120	67·8	67·4	91·5	92·2
180	71·3	71·0	95·7	95·6
300	75·0	75·0	—	—
480	76·6	78·1	—	—
1440	84·8	86·9	—	—
2880	95·0	93·9	—	—

* Assuming the composition to be: 52 % of an ester, $k = 75\times10^{-3}$; 20 % of the Neuberg ester, $k = 4{\cdot}2\times10^{-3}$; 28 % of aldose ester, $k = 0{\cdot}23\times10^{-3}$.

† Assuming the composition to be: 75 % of an ester, $k = 75\times10^{-3}$; 25 % of the Neuberg ester, $k = 4{\cdot}2\times10^{-3}$.

Table V. *Hydrolysis of hexosediphosphate in* N *HCl at 100°.*

	(0·454 mg. P per cc.)		Lohmann's values	
Time min.	Hydrolysis %	$k\times10^3$	Hydrolysis %	$k\times10^3$
5	23·3	23·0	22·3	21·9
10	36·8	16·8	36·8	17·9
15	44·9	11·9	44·9	11·8
25	54·4	8·2	53·8	7·6
40	61·5	4·9	61·3	5·6
60	68·8	4·6	66·7	3·0
90	79·1	5·8 } 4·6	75·8	4·6
120	83·5	3·4 }	80·9	3·4
180	90·2	3·8	87·3	2·9
300	96·6	3·8	93·5	2·4
480	99·4	4·2	95·5	0·87

The value of *k* for the phosphate group in position 1, calculated from our data, is 52×10^{-3}.

The amounts of hydrolysis for the two esters are shown in Table IV together with the values calculated on the assumptions stated above. The hydrolysis of the hexosediphosphate is shown in Table V, Lohmann's values for this ester being also shown for comparison. It will be seen that there is close agreement up to about 60 % hydrolysis, but that our values are somewhat higher than Lohmann's for the later stages. The final values of k are in reasonable agreement with that found for the fructosemonophosphate isolated from the products of fermentation. These results will be discussed more fully in a subsequent paper. The hydrolysis of preparation A (before oxidation) was also studied in 0·1 N HCl; the initial rate was approximately one-tenth of that in N HCl so that the new ester does not show the abnormal behaviour observed in the case of the two aldosemonophosphates.

Removal of the fructosemonophosphate by fractional hydrolysis with HCl. The nature of the first barium salt (A) was also investigated by fractional hydrolysis with HCl. 0·4 g. of the salt was heated in 0·1 N HCl at 100° until 43 % of the total phosphate had been hydrolysed. Another portion (0·6 g.) of this salt was heated in N HCl until hydrolysis reached 66 %. The residual hexosemonophosphates were then recovered in the form of their barium salts which were freed from inorganic phosphate and analysed. The results are given in Table III. The product after 43 % hydrolysis was dextrorotatory but still contained about 50 % of fructosemonophosphate; after 66 % hydrolysis the proportion of fructose esters had been reduced to 10 % or less, while the iodimetric value corresponded with 84 % of the total hexose; the $[\alpha]_{5461}$ was $+15{\cdot}8°$. From these facts it would appear probable that the aldose esters, which formed 28 % of this hydrolysis product, consisted chiefly of glucosemonophosphate with possibly a small proportion of mannosemonophosphate. The amount of material available did not allow further investigation at this stage.

Discussion.

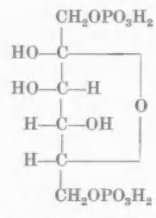
The experimental results, which have been described, furnish additional evidence of the tendency of the hexosephosphoric esters to undergo intramolecular change during hydrolysis by enzymes. They do not suffice to show whether this change is limited to the two terminal carbon atoms of the hexose molecule or whether a more radical alteration may also take place. It is shown that removal of both phosphoric acid groups by the agency of bone phosphatase, at low temperatures (25°) and in neutral solution, leads to the production of a mixture of reducing sugars, probably containing fructose and glucose, though, from the specific rotation, it would appear that other sugars, or sugar derivatives having reducing power or optical activity, may also be present. The relative proportions of aldoses and ketoses in the mixture vary considerably but the cause of these variations is not at present clear.

The investigation of the hexosemonophosphates produced by partial hydrolysis of the diphosphate under similar conditions has also given proof of the increased lability of the terminal groups of the hexose molecule during removal of the phosphate radical. The formation of an aldosemonophosphate, probably glucose-6-phosphate, and of fructose-6-phosphate, is in conformity with the results obtained by Neuberg and Leibowitz with phosphatases of different origin; but the formation of the third product, the laevorotatory ester, has not previously been observed. Our attempts to isolate this new ester in pure condition have been, so far, only partially successful. It was possible to eliminate the aldose esters by oxidation with bromine; but the residual ester still contained

the Neuberg fructosemonophosphate, to the extent of 25 %. The composition of this product was, however, determined with considerable certainty by studying its hydrolysis in *N* HCl, and it was thus possible to calculate what the properties of the pure ester would be. The calculated values (based on the analytical results for salt No. 6) are shown in Table III, No. 9. These properties, together with the very high rate of hydrolysis in *N* HCl at 100° ($k = 75 \times 10^{-3}$) strongly suggest that the new ester is fructose-1-phosphate and is probably a derivative of normal fructose.

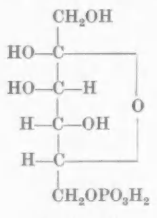
The proportions (28 : 20 : 52) in which the three monophosphoric esters, glucose-6, fructose-6 and fructose-1, were formed in product A of Exp. 9 further suggest that bone phosphatase, under the conditions of these experiments, hydrolyses both phosphoric acid groups at similar rates, and that during the removal of the group from position 1, intramolecular change occurs, resulting in the formation of glucose-, fructose- and possibly mannose-6-phosphate.

Hexosediphosphate

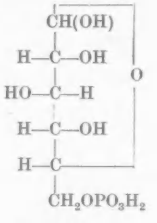


γ-fructose-1 : 6-diphosphate. 1 : 6-diphospho-fructofuranose.

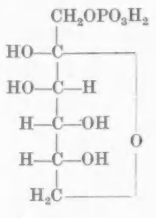
Products of hydrolysis by bone phosphatase



γ-fructose-6-phosphate. 6-phospho-fructofuranose.



glucose-6-phosphate. 6-phospho-glucopyranose.



fructose-1-phosphate. 1-phospho-fructopyranose.

It is quite possible that the laevorotatory fructose-1-phosphate occurs in the products of alcoholic fermentation but has hitherto escaped detection owing to its unstable character. The somewhat variable values which have been given by different workers for the specific rotation of the Neuberg ester may perhaps be explained by the presence of the new ester in very small proportions. Work on this compound is being continued and it is hoped to overcome the difficulties of its isolation in the pure state.

Summary.

1. The hydrolysis of hexosediphosphate (1 : 6-diphosphofructofuranose) by bone phosphatase at 25° and p_H 7·0 gives rise to a mixture of aldoses and ketoses in varying proportions. This confirms the previous findings of Martland and Robison.

2. The partial hydrolysis of hexosediphosphate, under similar conditions, gives a mixture of aldose- and fructosemonophosphates.

3. The chief aldose ester is probably glucose-6-phosphate.

4. The fructose esters consist of fructose-6-phosphate (Neuberg ester) together with a strongly laevorotatory compound, which is very rapidly hydrolysed by *N* HCl at 100°, $k = 75 \times 10^{-3}$. This product formed about 50 % of the total monophosphate fraction.

5. The new ester has not yet been obtained in pure condition, but its properties have been determined from those of the purest preparation, which was

free from aldose esters but still contained 25 % of the Neuberg ester. The calculated value of $[\alpha]_{5461}$ is $-35°$ for the barium salt. Its H. and J. reducing power is only two-thirds of that of the three known monophosphates.

6. The properties of the ester strongly suggest that it is fructose-1-phosphate, probably a derivative of normal fructose.

(I would here acknowledge my indebtedness to Dr M. G. Macfarlane for her help in completing these experiments, which were unhappily interrupted by the death of Miss Macleod in December 1931. R. R.)

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XLII. DETERMINATION OF VITAMIN A IN OILS BY A SPECTROPHOTOMETRIC METHOD.

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(*Received December 28th, 1932.*)

The present communication records experiments on the quantitative determination of vitamin A by the spectrophotometric method. The constant presence of a band at 3280 Å. in the absorption spectra of substances which restore the normal growth of rats deprived of vitamin A has suggested to many authors the possibility of measuring the quantity of vitamin A present by the intensity of this absorption band.

The existence of the band was noted by Morton and Heilbron [1928]; more recently, Coward, Dyer, Morton and Gaddum [1931] have compared the value of the biological test they employed with spectrographic and colorimetric determinations.

Norris [1931] has furthermore published results which are evidently not in favour of physical measurements.

Having elaborated a simple and accurate spectrophotometric method, we have been able to study the general spectra of oils, and were thus led to examine the conditions under which the absorption at 3280 Å. could show the quantity of vitamin A in a product.

Methods.

The principle of the technique employed, the details of which are given in another paper [Chevallier and Dubouloz, 1932], is the following. A continuous ultra-violet radiation, emitted by a hydrogen tube, is dispersed by a quartz monochromator. At the end of the monochromator is placed a potassium photoelectric cell covered with a fluorescent substance. The photoelectric current, generated by the light of the fluorescence, is amplified and measured by a galvanometer. There is a constant ratio between the ultra-violet radiation, the intensity of the fluorescence and the photoelectric current. Direct readings on the galvanometer give with consistent accuracy the value of the absorption of a substance placed in the radiation between 4000 and 1800 Å.

Our determinations on oils have been carried out with pure hexane as solvent, and the value of the absorption $\log I_0/I$ is always expressed in concentrations of 1/100 studied in a thickness of 1 cm.

A great number of measurements on plant and animal oils showed us that each oil, according to its nature, has a characteristic absorption spectrum. We determined the characteristic absorption curves for olive oils, maize oils and cod-liver oils. The differences of absorption for different oils of the same nature proceed from factors which we have succeeded in analysing.

If free acids be present, absorption bands are found, one of these having a maximum at 3175 Å. The pigment of certain oils also gives an absorption, mostly at 3300 Å. For the estimation of vitamin A we therefore used oils containing the minimum possible quantity of pigment and of free acid; if necessary, these substances were first extracted with alcohol.

EXPERIMENTAL DATA.

In order to compare the biological and the physical tests for vitamin A, we adopted the following scheme.

The absorption spectrum of a pure cod-liver oil being determined and $\log I_0/I$ calculated:

$$\log I_0/I \text{ (conc. 1 \%, thickness 1 cm.)} = 0{\cdot}950 \text{ at } 3280 \text{ Å.},$$

we prepared three dilutions of it in paraffin oil so that the first contained per drop, 5 mg. of the original oil, the second 7·5 mg. and the third 10 mg.

Three groups of young white rats of seven rats each have been fed with the following diet deprived of vitamin A:

Pure caseinogen washed with warm alcohol	15 %
Dextrinised rice starch	73 %
Dried yeast	8 %
Salt mixture (Osborne and Mendel)	4 %

When growth ceased some animals from each group were kept as controls and feeding with the above diet was continued until their death.

To each of the other animals we administered daily a drop of one of the diluted oils. For the first group: series 0, a drop of the first dilution; for the second group: series 60, a drop of the second dilution; for the third group: series 20, a drop of the third dilution.

A rapid gain in weight was noticeable in the three series. The increase in weight was roughly proportional to the quantity of oil received by the animals. The curves (Figs. 1, 2 and 3) show the average increase in weight of animals of the same series (series of 7 rats).

By this experiment it was possible to establish a precise ratio between the biological titration of our oil and its absorption at 3280 Å. This oil called sample 1 has been used as a reference.

In order to study the agreement between the absorption and the biological test of various oils, we selected from among the oils examined by means of our spectrophotometric method two different samples both showing an absorption considerably higher than that of sample 1. Of these the first, sample 13, gave an absorption:

$$\log I_0/I \text{ (conc. 1 \%; thickness 1 cm.)} = 12{\cdot}5,$$

that is thirteen times greater than that of sample 1, and the second, sample 4, gave an absorption of 3·8, four times greater than that of sample 1.

In order to carry out the biological tests under comparative conditions, the oil investigated was diluted with paraffin oil and fed to a group of rats.

The sample 13 being considered according to the physical measurement only to be thirteen times more active than sample 1, was diluted so as to correspond in absorptive power with the solution of which 1 drop contained 5 mg. of sample 1; one drop of this dilution was given each day to the rats of group 30. Group 40 received a dilution of sample 13, corresponding in absorptive power with the solution of which 1 drop contained 10 mg. of sample 1.

A similar procedure was adopted with sample 4, the activity of which was considered to be four times as great as that of sample 1; a dilution was prepared corresponding to a quantity of 7 mg. of sample 1 per drop. Another dilution corresponding to 10 mg. of sample 1 was also prepared and given to the rats of group 70.

In each of these series, as before, a few animals were kept as controls and did not receive any oil: they died after having shown visible signs of vitamin A deficiency.

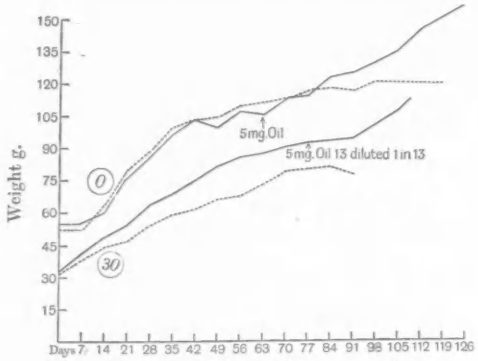

Fig. 1. Groups 0 and 30. Growth curves of rats.
——— rats receiving vitamin A; ------- controls.

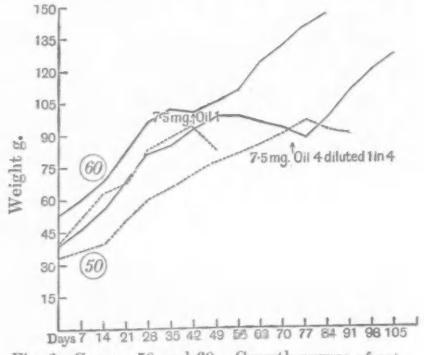

Fig. 2. Groups 50 and 60. Growth curves of rats.
——— rats receiving vitamin A; ------- controls.

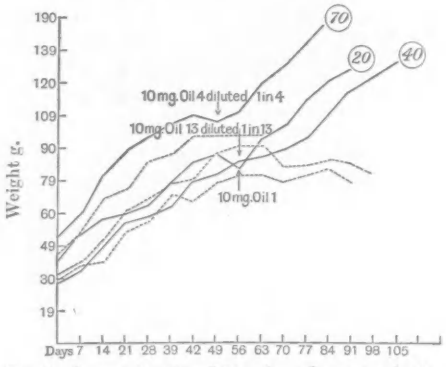

Fig. 3. Groups 20, 40 and 70. Growth curves of rats.
——— rats receiving vitamin A.; ------- controls.

Results.

The results of our experiments are reproduced in Figs. 1, 2, 3 and in the Table below:

No. of group	Sample	Absorption of sample at 3280 Å. log I_0/I 1 % 1 cm.	Dilution of the samples	Daily dose per animal mg.	Gain in weight per week (mean) g.	Difference %
0	1	0·950	1	5	5·11	—
30	13	12·5	1/13	5	4·55	11
60	1	0·950	1	7·5	7·84	—
50	4	3·8	1/4	7·5	7·77	1
20	1	0·950	1	10	9·10	—
40	13	12·5	1/13	10	7·91	13
70	4	3·8	1/4	10	9·30	2

Discussion.

Our results show a remarkable agreement between the values obtained by the biological and spectrophotometric methods. Our biological titration was carried out as carefully as possible, and, but for a few cases, the animals showed a gain in weight proportional to the quantity of oil they received. We attribute this to the fact that the animals in each of the groups were of the same litter, and that when showing vitamin A deficiency they received the oil *per os*, each day by means of a pipette. The fact that the rats were kept singly in separate cages, from the beginning of each experiment, seems also to be a factor in improving the results of the biological test. Our titration of sample 1 corresponded to three different doses (5 mg., 7 mg, and 10 mg.) so as to obtain greater accuracy in the measurement. We also used quantities too strong for interpretation by Sherman's "rat unit" method: average gain of weight 5·11 g. weekly for 5 mg. We chose this slightly greater quantity because we did not know what results samples 13 and 4 would give us. In this way, if the biological activity of these samples had been lower than what was expected from the physical titration, we should still have been able to establish a comparison.

In fact, the differences between the biological and the physical values obtained for samples 13 and 4 do not exceed 13 %.

We believe, therefore, that it is legitimate to employ the physical method for estimating the quantity of vitamin A in an oil. But as we have noted in another publication [Chevallier, Guillot and Chabre, 1932] the content of free acid in a product is very important. Some samples showing at 3280 Å. only an inflection in their absorption spectrum (see Fig. 4) showed, after their free fatty acids were removed by washing with alcohol, an absorption spectrum with the characteristic band at 3280 Å. We consider that the intensity of this band measures the quantity of vitamin A in a product, always providing that the presence of excess of pigment or of free acids does not interfere with the absorption.

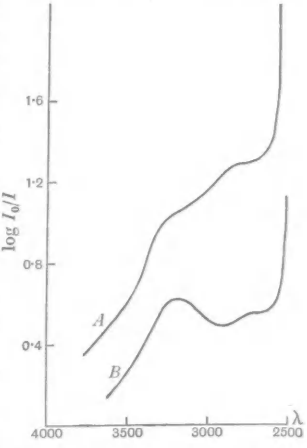

Fig. 4. *A*, Absorption curves of an acid oil; *B*, the same product washed in alcohol.

We think that the spectrophotometric method is interesting not only because it is more convenient and more rapid than the biological method, but also because it allows the direct assay of products which might contain a large quantity of vitamin. It is well known that the biological test is only valuable if the quantity of the sample administered to the rats corresponds roughly to the quantity of vitamin necessary for the maintenance of normal growth. It is therefore difficult to titrate products which contain large amounts of vitamin. One has then to make a great number of estimations with different dilutions, on a great many groups of animals. This difficulty does not exist with the physical method.

We have not spoken so far of the colorimetric method of determination by $SbCl_3$. We tried it on each of our samples, but the results were so inconsistent, that we think it advisable to use it only for qualitative determinations. Most other authors are of the same opinion.

SUMMARY.

We have applied a spectrophotometric method, which permits a very accurate measurement of the ultra-violet absorption given by a substance, to the measurement of the intensity of absorption at 3280 Å. of different samples of cod-liver oil.

The examination of a great number of oils of different origins showed us, that, besides the presence of vitamin A, the free acidity of the oil and in certain cases its pigment (if very concentrated) must be taken into account in consideration of the absorption in the neighbourhood of 3280 Å.

When the free acid content of the oil is low, the results of the physical and biological tests agree fairly well, the differences not exceeding the usual experimental errors inherent in biological methods.

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XLIII. VITAMIN C AND THE SUPRARENAL CORTEX.

II. LOSS OF POTENCY OF GUINEA-PIG SUPRARENALS IN SCURVY. WITH NOTES ON A METHOD FOR DETERMINING ANTISCORBUTIC ACTIVITY (HEXURONIC ACID) BY CHEMICAL MEANS.

By LESLIE JULIUS HARRIS and SURENDRA NATH RAY.

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(*Received December 31st, 1932.*)

In Part I [Harris and Ray, 1932] it was shown that ox suprarenals have a very high antiscorbutic activity, commensurate with their richness in hexuronic acid. The principal purpose of the present paper is to describe results already briefly alluded to in Part I, showing that the suprarenal gland of the normal guinea-pig has an antiscorbutic activity similar to that of the ox, and that this activity disappears with the development of scurvy. A method is also described for estimating hexuronic acid chemically, and it is shown that the hexuronic acid contents of orange juice, lemon juice and other sources accurately account for their observed antiscorbutic activity.

Experimental.

Harris's curative method [Harris, Mills and Innes, 1932; Harris and Ray, 1932] was used. The whole glands were fed in this case (instead of cortex only as in the experiments with ox suprarenals), with no apparent ill effects. The glands from normal and scorbutic guinea-pigs were fed at the two levels of 0·5 g. and 1·0 g., and the results were compared with those given by orange juice fed as standard at the two levels of 1·5 cc. and 3 cc., and with negative controls (basal diet only). In order to obtain a sufficient fresh daily supply of suprarenals from scorbutic guinea-pigs, large numbers of animals had to be placed on a scurvy-producing diet at daily intervals at a suitable length of time before the beginning of the assay. During the course of the determination, some of these animals, then suffering from scurvy, were killed daily and their suprarenals fed with as little delay as possible to the test animals. The suprarenals were prepared for feeding in the manner described in Part I. The same procedure was used for testing the suprarenals of normal guinea-pigs except that the diet of the latter was supplemented with cabbage (10 g. *per diem*).

Result. 0·5 g. of normal guinea-pig suprarenal was found to be approximately equivalent in antiscorbutic activity to 1·5 cc. of orange juice, and 1·0 g. of suprarenal to 3·0 cc. of orange juice. In the case of the suprarenals from scorbutic guinea-pigs, no antiscorbutic action could be detected even when fed at levels of 1 g. per day. Thus, animals receiving 1·0 g. or 0·5 g. of the scorbutic suprarenal

lost weight at the same rate as the negative controls (see Fig. 1); and no differences were apparent in the survival periods (Table I) or in the degree of severity of the scurvy, as determined at autopsy.

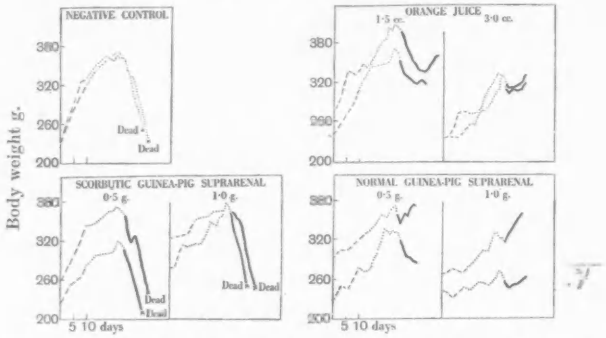

Fig. 1. – – – Preliminary period: scorbutic basal diet supplemented with cabbage.
. . . Depletion period: basal diet alone.
—— Experimental curative period.

Table I. *Survival periods, days (counting from beginning of depletion period).*

Negative controls	24, 22, 21	(av. 22·3)
Scorbutic suprarenal, 0·5 g. per day	24, 22	(av. 23)
Scorbutic suprarenal, 1·0 g. per day	26, 19	(av. 22·5)

Comments. The activity of the suprarenal of the guinea-pig is seen to be roughly three times as great as that of orange juice (wet weight). A similar value was found for the cortex of the suprarenal of the ox [Harris and Ray, 1932]. It follows therefore that weight for weight the whole suprarenal of the guinea-pig tends to be somewhat more active even than the whole suprarenal of the ox (in which species the medulla appears to be devoid of hexuronic acid).

Experiments are at present in progress to determine the antiscorbutic activity of the suprarenals of a number of dogs which have been maintained without ill effects for upwards of 7 months on a diet free from all vitamin C. Similar tests are being done on rats similarly kept for prolonged periods on vitamin C-free diets. Since these species are able to dispense with the need for vitamin C in their diet, it was of interest to compare their behaviour with that of guinea-pigs. Our colleague Dr Thomas Moore has examined the suprarenals for their hexuronic acid activity *in situ*, by the silver staining method of Szent-Györgyi [1928]. He has shown that whereas the suprarenals of normal guinea-pigs stain deeply with silver and lose this property after the animals have been kept for about 8 days on a scurvy-producing diet, the suprarenals of rats or dogs on the other hand continue to stain intensely even when there is no vitamin C in the diet [see Moore and Ray, 1932]. We have reached the same conclusion, determining the hexuronic acid chemically (see below).

Correlation between antiscorbutic activity and hexuronic acid content.

It was shown in Part I that the observed antiscorbutic activity of ox suprarenals could be accurately accounted for on the basis of their apparent hexuronic acid content. The relative antiscorbutic activities of ox suprarenals and orange

juice were proportional to the amount of hexuronic acid recoverable from the two sources. This was regarded as evidence in favour of the theory that hexuronic acid is identical with vitamin C. Additional arguments were also given in support of this view, but it was pointed out that further work would be needed before it could be regarded as definitely established that the activity of specimens of hexuronic acid was due to the main constituent and not to some undetected contaminant. A definite conclusion could only be reached by determining whether there existed a sufficiently constant and extended parallelism between antiscorbutic activity and hexuronic acid. One of the first desiderata to this end is to have a reliable method for estimating hexuronic acid chemically. We have carried out some experiments in this direction, as a result of which we have been able to show that the hexuronic acid content of orange juice, like that of suprarenals, is in accord with the theory that hexuronic acid and the vitamin are identical[1].

Prof. Szent-Györgyi suggested to us that the most hopeful method to try would be a rapidly carried out titration in acid solution either with iodine or dibromo-indophenol. The chief objection to the former method seemed to be that a considerable number of naturally occurring substances would reduce the iodine slowly, while glutathione would certainly reduce it rapidly, and hence the method would be inapplicable to the many natural sources containing glutathione. However, it gives theoretically perfect values with isolated hexuronic acid, and it seemed worth while to try it out, with various modifications, on certain sources, such as orange juice, known to contain little or no glutathione.

Unsatisfactory results with iodine titrations. Even with fresh orange juice, iodine titration in acid solution measures an appreciable amount of material in addition to hexuronic acid (judging the content of the latter from our later results), and it soon became apparent that the method was far too unspecific to give any reliable index. Orange juice which had been boiled and aerated to destroy the hexuronic acid and the antiscorbutic potency was found to show an

Table II. *Titration of specimens of fresh lemon or orange juice with standard iodine solution* (0·00895 *N*).

	Mg. of iodine reduced	Hexuronic acid equivalent, mg. per cc. of juice
Lemon juice, 5 cc.	3·94	
"	3·93	
" (in presence of 2 drops of glacial acetic acid)	3·91	0·54
" (by back titration method, KI and $Na_2S_2O_3$)	3·75	
Orange juice, 5 cc.	6·36	
" (in presence of 4 drops of glacial acetic acid)	6·30	0·87
" (by back titration method, KI and $Na_2S_2O_3$)	6·05	

almost undiminished titre (Tables II and III). It would appear from this that while the hexuronic acid is oxidised, other reducing substances are simultaneously formed in the orange juice under these conditions [*cf.* Zilva, 1930]. Control tests

[1] Svirbely and Szent-Györgyi [1932] have already expressed the belief that "the hexuronic acid present in lemon juice has the same activity as the lemon juice itself," but they had no precise information either as to the minimum dose of hexuronic acid or the hexuronic acid content of lemon juice. The latter was assumed to be about 0·33 mg. per cc., or, in an earlier paper [Szent-Györgyi, 1928], 0·25 mg. per cc. (for orange juice)—*i.e.* about one-half the value we find in the present paper. Not more than 0·1 mg. has actually been isolated per cc., representing one-third to one-sixth of the amount thus calculated to be present.

with various reducing agents showed that a number of these do in fact readily reduce iodine in acid solution, including catechol, tannic acid, quinol, pyrogallol, protocatechuic acid and solutions of glucose or fructose which had been boiled for 2 hours. In the case of fresh lemon juice, iodine titration in acid solution gave an approximately accurate measure of the hexuronic acid content, judged by our later results.

Table III. *Iodine titration values of orange juice after boiling and aeration.*

	cc. of 0·00818 *N* iodine solution required by 5 cc. of the juice (at natural acidity of juice)
Orange juice, before treatment	5·1
" boiled and aerated 30 mins.	5·6
" " " 60 "	5·8
Orange juice, before treatment	5·0
" aerated 30 mins.	4·6
" " 60 "	4·5
" " 95 "	5·15

Unsatisfactory results with 2:6-dichlorophenolindophenol in neutral solution. We next tried the use of this indicator in the manner described by Tillmans *et al.* [1932]. The procedure, in outline, is to titrate with the indicator until it is no longer reduced, the solution being first adjusted to near neutrality, by addition of sodium acetate, for which purpose the p_H colour change of the indicator itself is utilised. Here again the method broke down when tested by the titration values given by aerated orange juice (Tables IV and VIII); and it was found also that the indicator was reduced rapidly by pyrogallol or reduced glutathione and more slowly by catechol or tannic acid. The specificity of Tillmans' method therefore cannot be relied upon under certain conditions.

Table IV. *Titration of aerated orange juice with 2:6-dichlorophenolindophenol.*

	cc. of indicator solution required
5 cc. of orange juice, untreated (titrated at natural p_H of juice)	6·03
" aerated for 40 mins. (titrated at natural p_H of juice)	5·67
" " 60 " " "	5·7
" " 100 " " "	5·7
2 cc. of orange juice, untreated (neutralised with 25 cc. 20 % Na acetate)	2·5
" aerated for 60 mins. (neutralised with 25 cc. 20 % Na acetate)	2·5

Method adopted. A method depending on the use of the same indicator was eventually worked out which, when tested against various naturally occurring reducing agents, other than hexuronic acid (including pyrogallol, catechol, tannic acid, quinol, reduced glutathione and fresh and boiled solutions of glucose, fructose or sucrose[1]), was found to give consistently negative results, and which behaved satisfactorily with test solutions of hexuronic acid partially inactivated and with aerated fruit juice. There is little doubt that when applied for example to orange juice it can give results only little above the actual hexuronic acid content. The indicator is made up at a strength of about 0·01 *M*. To carry out a

[1] Pyrogallol and quinol reduce the indicator very slowly under these conditions, while with boiled sucrose or fructose solutions the colour is discharged on standing for some time: this behaviour is so different from that of hexuronic acid as not to affect the accuracy of the method.

titration the solution to be analysed is first brought to p_H about 2·5 (measured preferably by the hydrogen electrode) through the addition of a few drops of strong acetic acid. To a suitable volume of the solution (*e.g.* 5 cc. for orange juice) indicator is rapidly run in until it is no longer reduced. At first we experienced some difficulty in determining this somewhat indistinct end-point, especially when working with coloured solutions such as orange juice; but it was found that the difficulty could be entirely overcome by making use of some additional chlorophenolindophenol, used as external indicator, and a sharp end-point was reached. (The indicator is red, instead of blue as in Tillmans' method.) It has since been found that, for still greater precision, it is preferable to carry out the estimation by taking a measured volume of indicator solution and titrating it with the unknown [Birch, Harris and Ray, 1932]. To give the result in terms of mg. of hexuronic acid the indicator solution has to be standardised against a given solution of hexuronic acid, which in turn has to be standardised for its true hexuronic acid content. The last operation is readily carried out by means of an iodine titration, it being necessary only to remember that 1 molecule of hexuronic acid reduces 2 atoms of iodine. The procedure is best made clear by an actual instance. In the example cited (Table V) iodine titration showed that the

Table V. *Standardisation of hexuronic acid against iodine, and indicator solution against hexuronic acid.*

10 cc. of hexuronic acid solution (containing 1·72 mg. of a specimen of hexuronic acid) were titrated—

(1) against 0·00677 *N* iodine solution:
I required = 2·31 mg.;
Hence hexuronic acid present = 1·60 mg.;

(2) against indicator solution:
Volume of indicator required = 2·7 cc.
Hence, 2·7 cc. of indicator solution ≡ 1·6 mg. of hexuronic acid
or, 1 cc. ,, ≡ 0·6 mg. ,,

specimen of hexuronic acid used for making the test solution contained only 93 % of actual (fully reduced) hexuronic acid. Titration of this standardised hexuronic acid solution against the indicator solution now showed that 1 cc. of indicator solution was equivalent to 0·6 mg. of true hexuronic acid. Next titration of orange juice with this same indicator solution (Table VI) gave the result

Table VI. *Estimation of hexuronic acid in orange juice by titration with standardised indicator in acid solution.*

2·0 cc. of fresh orange juice, reduced to p_H 2·59, required of standardised indicator 2·05 cc.
Hence 2·0 cc. of orange juice contain 1·2 mg. of hexuronic acid.

that 2 cc. of orange juice reduced 2·0 cc. of indicator. Hence it was calculated that 2 cc. of orange juice contain 1·2 mg. of hexuronic acid. Table VII shows that this titration method does not give anomalous results with aerated specimens of orange juice, like the methods described earlier. With most natural sources of the vitamin a preliminary extraction process with trichloroacetic acid, sometimes followed by decoloration, is necessitated. With the numerous plant and animal sources so far examined (including orange juice, lemon juice, cabbage, watercress, grape-fruit, pineapple, tomato, horseradish, several varieties of apple, ox suprarenal, ox liver, cow's milk) the hexuronic acid content so determined agrees exactly with that calculated from the antiscorbutic activity. A typical result for lemon juice is set out in Table VII, and further results and a more detailed discussion will be given in a later paper [Birch, Harris and Ray, 1933].

Table VII. *Estimation of hexuronic acid content of lemon juice.*

1 cc. of hexuronic acid solution (about 0·1 %) required	1·2 cc. of indicator solution 2·57 cc. of 0·00453 *N* I solution.
Hence 1 cc. of indicator ≡	0·8 mg. of hexuronic acid
2 cc. of lemon juice, required (at p_H 2·5)	1·4 cc. of indicator solution.
Hence 2 cc. of lemon juice contain 1·1 mg. of hexuronic acid.	

Table VIII. *Control tests on aerated and heated orange juice, comparing Tillmans's with new method.*

	Cc. of indicator required	
	Tillmans' method	New method
2 cc. of orange juice, before treatment	2·1	2·0 (≡1·16 mg. hexuronic acid)
" aerated for 2 hours	2·4	1·6
" boiled for 2 hours	1·9	0·7

Table IX. *Further control tests with aerated orange juice,* etc., *(titrating in acid solution).*

	Cc. of indicator required (varying strengths were used in the different experiments)
5 cc. of orange juice, before treatment	8·0
" made alkaline and aerated for 30 mins.	2·0
" " " 60 "	1·1
" " " 90 "	0·6
2 cc. of fresh orange juice (p_H reduced to 2·59)	2·05
" +15 cc. of water	2·0
" "	2·1
" made alkaline and aerated for 2 hours	0·15
5 cc. of hexuronic acid solution (containing 1 mg. of original preparation)	
Before treatment	2·2
After aeration for 15 mins. in alkaline solution	0·2
" 30 " "	0

Agreement between observed and predicted hexuronic acid content. 1 cc. of orange juice contains, according to our indicator titration results, 0·6 mg. of hexuronic acid or slightly under, or according to the iodine titration result less than 0·8 mg. (since the iodine reacts with a certain amount of additional material in fresh orange juice as well as the hexuronic acid). This value agrees well with that predicted on the basis of the relative antiscorbutic activities of hexuronic acid and orange juice, on the supposition that vitamin C is identical with hexuronic acid. Thus according to Harris, Mills and Innes's [1932] assay of hexuronic

Table X. *Hexuronic acid content of orange juice, as determined chemically and as calculated from antiscorbutic activities.*

Method	Hexuronic acid mg. per cc.
Determined by chlorophenolindophenol titration	0·6
Determined by iodine titration	<0·8
Calculated from curative tests	Between 0·4 and 0·66
Calculated from preventive tooth-structure tests	" 0·33 and 0·66
Calculated from assumption that minimum preventive dose = 1·0 mg. to 0·5 mg.	" 0·33 and 0·66
Probable value	0·5–0·6

acid by the curative method, 2 mg. of hexuronic acid were somewhat more potent than 3 cc. of orange juice but less potent than 5 cc. of orange juice. (Confirmatory results, using hexuronic acid from different sources and a highly repurified specimen are shown in Fig. 2.) That is, 1 cc. of orange juice is antiscorbutically equivalent to rather less than 0·66 mg. of hexuronic acid but more than 0·4 mg. Again, the minimum protective dose of hexuronic acid (*i.e.* corresponding with 3·0 cc. of orange juice) as determined by the microscopic tooth-structure method was greater than 1 mg. but less than 2 mg. [Harris, Mills and Innes, 1932] so that on this basis 1 cc. of orange juice is equivalent to between 0·33 and 0·66 mg. of hexuronic acid. Again Svirbely and Szent-Györgyi [1932] found that 1 mg. of hexuronic acid daily sufficed as a preventive (smaller amounts were not tried), and if this be regarded as the minimum daily protective dose (*i.e.* corresponding with 1·5 cc. of orange juice), then 1 cc. of orange juice is equivalent to 0·66 mg. of hexuronic acid. King and Waugh's [1932] claim to a minimum dose of 0·5 mg. is probably slightly underestimated and would lead to the rather lower value of 0·33 mg.; the true value is probably between the two.

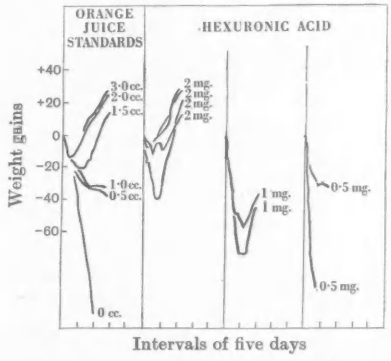

Fig. 2. Curative tests comparing hexuronic acid (weight curves of individual animals are shown) and orange juice (weight curves are averages of groups of four animals).

It may be concluded, then, that whatever slight adjustments in these values may be necessitated (as a result of more precise measurements of the activity of the acid, or the amount of it in orange juice) there can be no doubt that the antiscorbutic activity of orange juice agrees within very close limits with its hexuronic acid content (Table X). For lemon juice likewise (as for many other plant sources of vitamin C) we have found that indicator titrations lead to a similar conclusion. Moreover we have seen (Part I) that the same is true of suprarenal cortex; and, as our present results show, scurvy causes the disappearance of vitamin C from this organ, which coincides with a simultaneous loss of hexuronic acid. Again we have found that the destruction of hexuronic acid proceeds at a similar rate to that of its antiscorbutic activity. Investigations are also in progress in which antiscorbutic activity has been correlated with hexuronic acid content as measured by other indices as well as reducing reactions, and fuller details will be published later [Birch, Harris and Ray, 1933]. In conclusion therefore it may be claimed that an extensive quantitative correlation has already been proved between hexuronic acid and antiscorbutic activity.

SUMMARY.

The suprarenal of the normal guinea-pig has an antiscorbutic activity and hexuronic acid content similar to (or somewhat greater than) that of the ox. The activity disappears together with the hexuronic acid when vitamin C is withheld from the diet. A preliminary account is given of experiments contrasting this behaviour of the guinea-pig with that of other species such as the dog and rat, which can synthesise the vitamin when none is provided in their food.

A method of estimating hexuronic acid has been worked out, depending on modifications in the use of Tillmans's reduction indicator 2·6-dichlorophenol-indophenol. The hexuronic acid content of orange juice determined by this means accounts accurately for its observed antiscorbutic potency. A similar correlation has been established between hexuronic acid and vitamin C in the cases of lemon juice and various other sources, normal and scorbutic suprarenals, and during destruction by oxidation, *etc.*, and supports the theory of the identity of the two.

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XLIV. FURTHER OBSERVATIONS ON SURFACE FILMS OF OESTRIN DERIVATIVES AND OF PREGNANDIOL.

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The work described in this paper is a continuation of that described in a previous communication [Adam *et al.*, 1932]. It describes the results of surface pressure and surface potential measurements on various derivatives of oestrin, and of pregnandiol [*cf.* Marrian, 1929], spread in the form of unimolecular layers on the surface of aqueous solutions. Further deductions are drawn concerning the structure of oestrin.

It has now been shown by Marrian and Haslewood [1932, 1, 2] and Butenandt [1932, 1] that oestrin must have a four-ringed structure. In the previous paper of Adam *et al.* it was shown that if this were so, then only structures based on chrysene or naphthacene[1], or structures of these types, but containing five-membered rings (*e.g.* carbon skeletons of the types represented by formulae I–III and IV below), were possible. Trihydroxyoestrin $C_{18}H_{21}(OH)_3$ contains two adjacent hydroxyls; on oxidation it gives an 18 carbon atom dicarboxylic acid $C_{16}H_{19}(OH)(COOH)_2$ in which the ring containing the adjacent hydroxyls has been opened. Pyrolysis of this compound gives an anhydride $C_{16}H_{19}(OH)(CO)_2O$, [Marrian and Haslewood, 1932, 1, 2]. This probably indicates that the adjacent hydroxyls are either in a six-membered ring in the middle of the fused nucleus, or in a five-membered ring at the end of the nucleus. The surface film results [Adam *et al.*, 1932] suggested that the alcoholic hydroxyls were in one end ring, and that the phenolic hydroxyl was in the other end ring. There was however no definite evidence of the position of the phenolic hydroxyl.

Butenandt [1931] has shown conclusively that pregnandiol is a dihydroxylic sterol derivative, having one hydroxyl in position 3 and one secondary alcoholic hydroxyl in the side-chain, which contains only two carbon atoms.

Experimental.

The surface pressure (the outward force on a barrier in the surface dividing the film-covered surface from a clean water surface) was measured by the apparatus of Adam and Jessop [1926, 1] and Adam [1930, p. 37]. The substances were weighed out in small stoppered bottles, in quantities of about 1 mg., and about 2 cc. of a benzene (5 parts)-alcohol (2 parts) mixture added. The solution was dropped on to the surface from a calibrated dropping pipette, employing

[1] It was at the time suggested that 2 : 3-benzphenanthrene was also possible, but measurements made on models indicate that this is very improbable in view of the small cross-section of the molecule.

the corrections for evaporation described by Adam and Jessop [1926, 2]. The results are susceptible to an error of 5 %.

The films were examined for collapse by the method of Zocher and Stiebel [1930], in which a cardioid condenser is inserted through the bottom of the trough, giving intense dark-ground illumination of the surface. Collapse aggregates show up in this way as bright spots on a dark ground, whereas perfect unimolecular films show nothing.

The change in the electrical potential at the air-liquid interface was measured by a modification of the method recently published by Schulman and Rideal [1931], as described by Adam and Harding [1932]. Surface pressure and surface potential measurements were always made simultaneously. When dealing with slowly collapsing films surface pressure measurements were taken rapidly, and then surface potential measurements made at constant surface pressure, the pressure being kept constant by slowly decreasing the area occupied by the film. Then, in plotting the surface potentials, the areas per molecule could be read off from the surface pressure-area curve.

Results.

Experiments have been made on the Clemmensen reduction product of ketohydroxyoestrin, and on the reduction product of the semicarbazone of ketohydroxyoestrin, both of which have one phenolic hydroxyl; on a diol (dihydroxyoestrin) containing one phenolic and one alcoholic hydroxyl, obtained by hydrogenation of ketohydroxyoestrin and also on the diacetate and monomethyl ether of the diol, the alcoholic hydroxyl being the free one in the ether. The other compounds investigated were the diol with the benzene ring reduced (hexahydrodihydroxyoestrin) (*i.e.* with two alcoholic hydroxyls), pregnandiol, and the corresponding diketone. Results are also included for two sterols, calciferol and ψ-cholesterol, for purposes of comparison [Adam and Danielli, unpublished]. The results are shown in Figs. 1 and 2. All the measurements were made with films upon $N/100$ hydrochloric acid.

Fig. 1 shows the surface pressure results. Curves of the triacetyl derivative and the diacetyl derivative of the methyl ether of trihydroxyoestrin, in which the phenolic hydroxyl has been methylated, are included for purposes of comparison. These results have already been described [Adam *et al.*, 1932].

The Clemmensen reduction product and the reduction product of ketohydroxyoestrin semicarbazone give surface pressure curves which are identical within experimental error. The curve is practically linear above 5 dynes pressure and tends to a limit of 47 sq. Å. $\pm$ 2; below 5 dynes the surface pressure-area curve rounds off, tending to 50 sq. Å. $\pm$ 2. This curve shows a very considerable resemblance to that obtained with calciferol (vitamin D), which is included in Fig. 1, but the oestrin derivatives are rather less compressible. Ultramicroscopic examination revealed only a very slow collapse even at surface pressures as high as 20 dynes.

The diol obtained by an incomplete hydrogenation of ketohydroxyoestrin formed a gaseous film which was however too unstable even for surface pressure measurements. The corresponding derivatives in which the phenolic hydroxyl is methylated or both hydroxyls are acetylated give much more satisfactory results. The methylated derivative gives a moderately compressible film; the curve is practically linear above 4 dynes, the upper region tending to 33·5 sq. Å. $\pm$ 3; the lower region is rounded off to an unusually high degree, tending to about 40 sq. Å., and giving a constant vapour pressure above this area of about 0·2 dyne/cm.

There is no sign of such a transition as was observed with the derivatives of trihydroxyoestrin, which gave gaseous films at high areas. Films of this substance

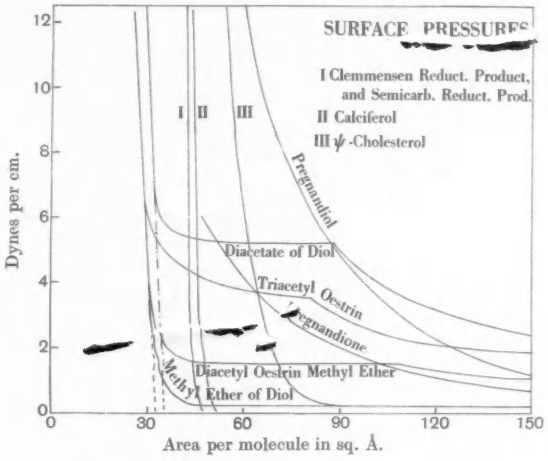


Fig. 1.

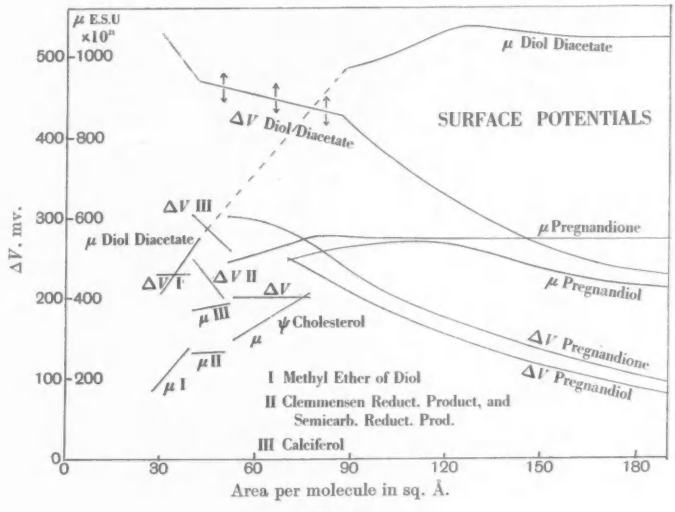


Fig. 2.

collapsed fairly easily. The acetylated derivative was much more stable and gave a surface pressure curve of the same type as was obtained with triacetyloestrin. At large areas the film is gaseous with rather smaller corrections to the perfect gas laws than are required with triacetyloestrin. On compression to 88 sq. Å. and

5·2 dynes/cm. a sharp break occurs in the curve, which becomes horizontal within experimental error over the range 88–42 sq. Å., in this respect differing from triacetyloestrin and diacetyloestrin methyl ether, both of which give curves sloping upwards slightly in this region as the area per molecule decreases. At the left-hand end of this region the curve rises sharply, giving a typical curve of a condensed film, which tends to an area of 35 sq. Å. ± 3 at no compression.

The films of the diol with the benzene ring reduced (hexahydrodihydroxyoestrin) were also gaseous, but were too unstable for measurements to be made.

Pregnandiol (formula VII) and pregnandione both give gaseous films, pregnandiol being the more perfect gas. Both substances collapse readily, pregnandione being less stable than pregnandiol. The remaining curve on Fig. 1 is that of ψ-cholesterol, a monohydroxylic sterol having a hydroxyl group in ring *B* at position 6 or 7 [formula of Rosenheim and King, 1932]. It is not a gaseous film, but is much more compressible than any of the substances previously mentioned excepting pregnandiol and pregnandione. It tends to an area of 80 sq. Å. ± 3 at zero compression, falling to 52 sq. Å. at a surface pressure of 15 dynes/cm.

Fig. 2 shows the changes in surface potential ΔV, due to the film and also the changes of μ, calculated from Helmholtz's equation,

$$\Delta V = 4\pi n\mu,$$

n being the number of molecules per sq. cm., and μ the average vertical component of the change in dipole moment of the part of the surface occupied by a molecule of the film, including the dipole moment of the film molecules themselves and all effects due to redistribution of water molecules and of ions in the underlying solution.

The surface potential (and consequently the μ) curves of the Clemmensen reduction product of ketohydroxyoestrin and of the reduction product of the semicarbazone of ketohydroxyoestrin are identical within experimental error. The surface potential increases linearly with decrease of area at areas less than 50 sq. Å. ± 2, and exhibits fluctuations, due to the presence of more than one phase, at greater areas. Over the non-fluctuating condensed region μ remains constant at $2{\cdot}65 \times 10^{-19}$ E.S.U. ± 0·05. In the case of calciferol ΔV is higher and increases less rapidly with area, the curve again being almost linear. The μ curve is almost linear, μ falling off slowly from $3{\cdot}8 \times 10^{-19}$ E.S.U. at 52 sq. Å. to $3{\cdot}7 \times 10^{-19}$ E.S.U. at 41 sq. Å. At areas greater than 52 sq. Å. ΔV fluctuates, owing to the presence of vapour and liquid phases in equilibrium.

It was impossible to make measurements on dihydroxyoestrin, but satisfactory measurements were obtained on the derivatives of this diol in which the phenolic hydroxyl had been methylated or both hydroxyls acetylated. With the methylated derivative the surface potential remained almost constant at all areas obtainable below 39 sq. Å.; at larger areas there were large fluctuations in ΔV. The surface pressure at 39 sq. Å. is still as high as 0·5 dyne, and it is a little surprising that ΔV should fluctuate at so high a pressure, where one would expect only one phase to be present; this phenomenon is frequently encountered in derivatives of the sterols. The μ curve is also linear, falling from $2{\cdot}4 \times 10^{-19}$ E.S.U. at 39 sq. Å. to $1{\cdot}8 \times 10^{-19}$ E.S.U. at 30 sq. Å. The surface potential curve of the acetyl derivative is similar to that of triacetyloestrin, excepting that over the range of constant surface pressure (42–88 sq. Å.) the potential fluctuates by ± 40 mv., whereas triacetyloestrin and diacetyloestrin methyl ether show no fluctuations. At areas above 88 sq. Å. ΔV falls off slowly with increasing area and fluctuations are certainly less than 5 mv., the film obviously being gaseous:

at 88 sq. Å. μ is $9{\cdot}7 \times 10^{-19}$ E.S.U., and it tends to a limit of $10{\cdot}5 \times 10^{-19}$ E.S.U. $\pm 0{\cdot}5$ at large areas. Measurements at areas below 42 sq. Å. are more difficult to make since the film collapses fairly rapidly; ΔV increases linearly with decreasing area, and μ falls from $5{\cdot}5 \times 10^{-19}$ E.S.U. at 42 sq. Å. to $4{\cdot}2 \times 10^{-19}$ E.S.U. at 30 sq. Å.

The films of pregnandiol and pregnandione are homogeneous at all areas. At any given area ΔV, and consequently μ, is higher for the diketone than for the diol. In both cases also μ appears to tend to a limiting value at large areas, $4{\cdot}1 \times 10^{-19}$ E.S.U. for pregnandiol, $5{\cdot}4 \times 10^{-19}$ E.S.U. for pregnandione. Pregnandione differs from pregnandiol in that there is very little change in μ until the area per molecule is brought below 80 sq. Å., after which μ falls off fairly rapidly, whilst with pregnandiol μ begins to increase on decreasing the area below 170 sq. Å., reaching a maximum of $5{\cdot}3 \times 10^{-19}$ E.S.U. at 110 sq. Å., and then falling off again.

The substance ψ-cholesterol gives a constant non-fluctuating value of ΔV over the whole range of 54–77 sq. Å., μ falling from $4{\cdot}1 \times 10^{-19}$ E.S.U. at the higher area to $3{\cdot}0 \times 10^{-19}$ E.S.U. at the lower area. At areas greater than 77 sq. Å. fluctuations in ΔV are encountered, as is usual when more than one phase is present.

Discussion.

Neither surface pressure nor surface potential measurements show any difference between the Clemmensen reduction product of ketohydroxyoestrin and the reduction product of the semicarbazone of ketohydroxyoestrin, so it seems fairly certain that the hydroxyl group must be in the same position in these two compounds. Any differences which may exist must be minor stereochemical differences in the configuration of the carbon skeletons, too small to affect either the shape of the molecule or the inclination of the hydroxyl group to the ring system. We see no reason however for supposing that these compounds are not identical[1]. The surface pressure curves of these two substances are similar to that of calciferol, whilst the surface potentials are quite different; a possible explanation of this may be that the hydroxyls are in similar positions in the two compounds, the potential differences being due to the hydroxyl being phenolic in the oestrin derivatives and alcoholic in the sterol. The small area per molecule and the incompressibility of these films indicate that the molecules must be standing on end and close-packed; consequently the hydroxyl must be in an end ring, since there would otherwise be no sufficiently strong water-attractive group next to the water to stabilise the film.

The derivatives of the diol obtained by an incomplete hydrogenation of ketohydroxyoestrin (dihydroxyoestrin) must also be standing on end and close-packed at small areas. In the close-packed position the alcoholic hydroxyl in the methyl derivative group must be next to the water, since an ether is considerably less water-attractive than a free alcoholic hydroxyl. The small area occupied by the acetylated derivative probably indicates that this substance when standing upright has the same orientation as the methylated derivative, *i.e.* it is standing upon the alcoholic end of the molecule. These two derivatives are thus standing the opposite way up to the two more completely reduced derivatives discussed in the previous paragraph; in this they resemble the derivatives of trihydroxyoestrin, triacetyloestrin and diacetyloestrin methyl ether [Adam *et al.*, 1932]. The limiting

[1] See footnote to section on preparations, p. 318.

areas at no compression for the close-packed curves are 33·5 ± 3 sq. Å. for the diol ether, 35 ± 3 sq. Å. for the diol acetate, and 32·5 ± 2 sq. Å. for the derivatives of trihydroxyoestrin. Thus it is fairly certain that the cross-sectional area of oestrin cannot differ much from 34 sq. Å. This is a somewhat small area for a chrysene type skeleton, judging from measurements made on models, but is just possible if a certain amount of interpenetration of adjacent molecules is permitted. The naphthacene type can be fitted into this area without interpenetration.

It was particularly noteworthy that dihydroxyoestrin diacetate gave a gaseous film at large areas, with a region of transition (88–42 sq. Å.) to the condensed state. As was pointed out in the earlier paper of Adam *et al.* there can be no doubt that in the gaseous state the molecules are lying flat and moving about more or less independently. The molecules will only lie flat if there is a water-attractive group at each end of the molecules; it is also known, from experiments on aliphatic and phenolic ethers, *etc.* [Adam and Dyer, 1924], that the water-attractive power of the methoxy-group is relatively small. Diacetyl oestrin methyl ether forms a gaseous film up to 1·5 dynes/cm.; the monomethylated diol does not form a gaseous film at any pressure above 0·2 dyne/cm. These compounds are identical in ring *A* differing only in ring *D*. The presence of fluctuations over the transition region of the diol acetate, which do not occur with the trihydroxyoestrin derivatives, may be due to the fact that the transition region is not reversible with the diol acetate but is reversible with the trihydroxy derivatives. The transition of the diol acetate is thus a two-dimensional collapse or non-reversible re-orientation, whilst with triacetyloestrin and diacetyloestrin methyl ether the re-orientation is reversible.

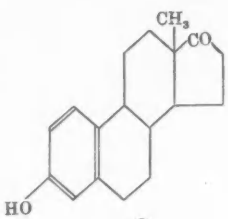
The gaseous nature of the films of pregnandiol (VII) and pregnandione is fully in agreement with the structure assigned to these compounds by Butenandt [1931], which ascribes to them a water-attractive group at each end of the molecule. The formula ascribed to ψ-cholesterol (VI), *i.e.* one with a hydroxyl in ring *B*, is also in good agreement with the film measurements. Measurements made on a model indicate that with a hydroxyl in ring *B* the molecule would lie flat at low pressures, occupying an area of the order of 80 sq. Å., and would readily be re-orientated by lateral pressure, so that the films would be expected to be much more compressible than, say, that of cholesterol, which cannot be so re-orientated. The experimental value of the limiting area is 80 sq. Å. ± 3. It should be noticed that there is a sharp distinction between the behaviour of this substance, which has a hydroxyl in ring *B*, and all the other substances, which have water-attractive groups in rings *A* and *D*.

The structure of oestrin.

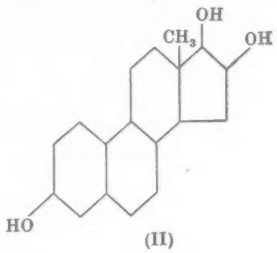
It has been shown [Adam *et al.*, 1932] that derivatives of trihydroxyoestrin give close-packed films tending to an area so small that the molecules can only be fitted in if they are standing on end so as to have the smallest possible cross-section. Since these films are stable it follows that the two adjacent alcoholic hydroxyls must be in an end ring of the carbon skeleton. This result has been confirmed by experiments on the methyl ether and the acetate of the diol obtained by an incomplete reduction of ketohydroxyoestrin, which give close-packed films in which the nucleus has the same orientation to the surface as it has in the trihydroxyoestrin derivatives. (Ketohydroxyoestrin can be obtained by dehydration of trihydroxyoestrin, so that the alcoholic group in the diol must be attached to one of the two carbons which carry alcoholic hydroxyls in trihydroxyoestrin.) There can thus be no doubt that the alcoholic hydroxyls in trihydroxyoestrin (and consequently the keto-group in ketohydroxyoestrin)

must be in an end ring. As was explained in the introduction this means that the terminal ring must be a five-membered ring (formula III or IV).

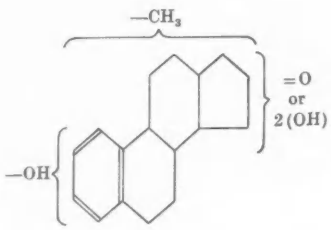
It is not possible to allocate positions to the hydroxyls in the five-ring, though it is obvious that they cannot be attached to the carbons shared with the



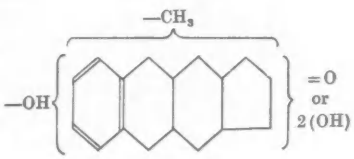
(I)



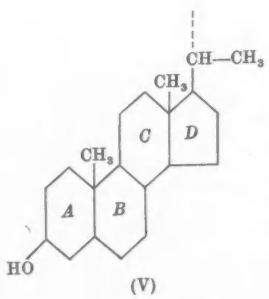
(II)



(III)

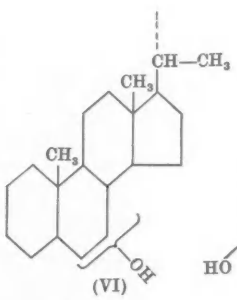


(IV)



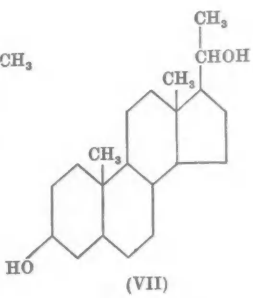
(V)

Calciferol
(Double bonds omitted)



(VI)

ψ-Cholesterol
(Double bond omitted)



(VII)

Pregnandiol

neighbouring ring, since in that case it would be impossible to obtain a dicarboxylic acid by oxidation of trihydroxyoestrin.

In the close-packed films which are obtained with derivatives containing only a phenolic hydroxyl this group must be in the water surface—otherwise the film would not be stable. The limiting area occupied by these films

(47 sq. Å.) is larger than that (34 sq. Å.) obtained when the molecule is orientated upon the five-membered ring, but comparison with other similar (but not necessarily identical) ring systems, *e.g.* calciferol (V), which has a hydroxyl in ring *A* and occupies 47 sq. Å., and ψ-cholesterol (VI), which has a hydroxyl in ring *B* and occupies 80 sq. Å., makes it quite evident that the phenolic hydroxyl must be in a terminal ring also. Consequently the ring at the end of the oestrin nucleus opposite to the five-ring must be the benzenoid ring. It is again impossible to place the hydroxyl exactly, but positions 2 or 3 are the more probable.

Nothing can be said as yet concerning the structure of the two centre rings of the nucleus; they may be either five-, six-, or seven-membered rings. The only evidence which exists at all is evidence by analogy with the sterols, in which case, assuming the structure of Rosenheim and King, the two centre rings would be six-membered rings; this view is supported, but not proved, by the X-ray measurements of Bernal [1932]. It cannot be too strongly emphasised that there is at the moment no satisfactory evidence that the oestrins are related to the sterols and bile acids. Since the preference for a modified chrysene type of skeleton, rather than the naphthacene type, is based upon the assumption that such a relationship does exist, the latter type of skeleton cannot be excluded. Nevertheless the fact that the only known condensed ring systems occurring in animal tissues, *i.e.* the sterols and bile acids, seem to possess the modified chrysene structure, does strongly suggest that the oestrins are of the same type, and provides a useful working hypothesis. On this basis formulae I and II may represent ketohydroxyoestrin and trihydroxyoestrin respectively, as has been suggested by Butenandt [1932, 2]. Butenandt however does not give evidence in support of the structures he suggests, and one can only conclude that he has placed the substituent groups by analogy with the sterol and bile acid formulae. The present authors are of the opinion that the most complete formula that can be advanced at the present time is the formula III, and even this is based on a certain amount of speculation.

Preparation of materials.

Reduction of ketohydroxyoestrin by Clemmensen's method[1]. The preparation used was the same as that previously described Marrian and Haslewood [1932, 1], M.P. 133–4°.

Reduction of ketohydroxyoestrin via *the semicarbazone*[1]. 109 mg. of ketohydroxyoestrin semicarbazone (M.P. 251–4°) were heated in a sealed tube with

[1] Butenandt, Störmer and Westphal [1932] stated that the Clemmensen reduction product of ketohydroxyoestrin was a non-phenolic compound of the formula $C_{18}H_{26}O$, whereas the reduction product obtained by the treatment of ketohydroxyoestrin semicarbazone with sodium ethoxide was a phenolic compound of the formula $C_{18}H_{24}O$. Marrian and Haslewood [1932, 1, 2] showed that the former compound was actually a phenol of the formula $C_{18}H_{24}O$ whose alkali salts were almost insoluble in water. Since Butenandt *et al.* had stated that a mixed melting-point of the two compounds showed a 5° depression, and since ketohydroxyoestrin when heated with hydrochloric acid gave a deep pink solution, Marrian and Haslewood were led to suggest that the hot acid in the Clemmensen reduction had caused an isomeric change. From the data given by Butenandt *et al.* it was argued that such a change might involve a change in position of the phenolic hydroxyl. In the course of the work described in the present paper, the reduction product from the semicarbazone has been prepared. It proved to have the same melting-point as the Clemmensen reduction product and a mixed melting-point showed no depression. No difference in alkali-solubility between the two compounds could be detected, nor was there any difference between the properties of their unimolecular films. There seems therefore no reason for supposing that two isomeric reduction products exist.

2 g. of the sodium ethoxide prepared by dissolving 1 g. of sodium in 15 cc. of ethyl alcohol, for 20 hours at 180–230°. The contents of the tube were washed out, diluted with water and saturated with carbon dioxide. The mixture was extracted with ether, and the ether washed with water and evaporated to dryness. The residue was distilled at 150–60°/0·01–0·02 mm. The crude crystalline sublimate was recrystallised from normal hexane and yielded 16·5 mg. of a white crystalline solid, M.P. 134–5°. A further 14·5 mg., M.P. 132–5°, was recovered from the mother-liquors.

Dihydroxyoestrin. $C_{18}H_{22}(OH)_2$. 100 mg. of ketohydroxyoestrin were dissolved in ethyl alcohol and shaken with Adams and Schreiner's platinum oxide catalyst with hydrogen at a pressure of 4 atmospheres for 36 hours. Two further additions of catalyst were made during the course of the hydrogenation. The alcoholic solution was filtered from the catalyst, evaporated to dryness and the residue heated with dilute sodium hydroxide. The insoluble material was filtered off and the filtrate saturated with carbon dioxide. The precipitate which formed was filtered off, washed with water and recrystallised from benzene. 51 mg. of white needles, M.P. 174–6° were obtained.

(Found: C, 78·40; H, 8·90 %. $C_{18}H_{24}O_2$ requires: C, 79·36; H, 8·89 %.)

Dihydroxyoestrin diacetate. 10 mg. of dihydroxyoestrin were heated with 3 cc. of acetic anhydride for 3 hours. After cooling the mixture was diluted with water and the gummy precipitate filtered off. The crude acetate could not be induced to crystallise from any solvent. It was therefore purified by boiling in alcoholic solution with charcoal and then by sublimation at 120°/0·01 mm. This process was repeated and the final product obtained by precipitating the alcoholic solution of the sublimate with excess of water; M.P. 120–2°.

(Found: C, 73·82; H, 8·02 %. $C_{18}H_{22}(O.OC.CH_3)_2$ requires: C, 73·14; H, 7·92 %.)

Dihydroxyoestrin monomethyl ether. 18·1 mg. of dihydroxyoestrin were shaken with 10 cc. *N* sodium hydroxide and 0·2 cc. of dimethyl sulphate for several hours. The crude product was filtered off, washed with water and dried. As in the case of the acetate, purification could only be effected by repeated boiling with charcoal in alcoholic solution and vacuum distillation. The final product, obtained by precipitating the sublimate from alcoholic solution with water, melted at 91–4°.

(Found: C, 79·32; H, 9·25 %. $C_{18}H_{23}O(OCH_3)$ requires: C, 79·66; H, 9·16 %.)

Hexahydrodihydroxyoestrin. $C_{18}H_{28}(OH)_2$. The fraction of the total products of hydrogenation of ketohydroxyoestrin which was insoluble in aqueous alkali was distilled at 180–200°/0·01 mm. The distillate was then heated with aqueous alkali to remove any traces of phenolic substances and finally recrystallised twice from benzene. 5 mg. of white needles, M.P. 208–10°, were obtained.

(Found: C, 77·3; H, 10·81 %. $C_{18}H_{28}(OH)_2$ requires: C, 77·64; H, 10·86 %.)

Better yields of the hexahydro-compound were subsequently obtained by hydrogenating in glacial acetic acid solution. The material used in the surface film experiment was prepared in this manner; M.P. 209–211°.

SUMMARY.

Surface pressure and surface potential measurements have been made on a number of derivatives of oestrin and on pregnandiol and pregnandione. The Clemmensen reduction product of ketohydroxyoestrin and the reduction product of the semicarbazone of ketohydroxyoestrin gave identical results. If Butenandt's view that these are different is correct, the differences must be concerned

with minor variations in the stereochemical configuration of the carbon skeleton.

It is concluded that one end ring of the oestrin nucleus is a five-membered ring, containing the two alcoholic hydroxyls of trihydroxyoestrin and the keto-group of ketohydroxyoestrin. The other end ring is benzenoid and contains the phenolic hydroxyl, most probably in position 2 or 3.

The results obtained with pregnandiol and pregnandione are in full agreement with the structures assigned to these compounds by Butenandt.

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